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TITLE: A Solid Support Synthesis and Novel Conjugation Methods of Breast Tumor Associated Antigen: Toward the Development of Cancer Vaccines

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The immunological study of globo-H, a breast tumor associated carbohydrate antigen, has been producing promising results. As the demand for this antigen grows, the efficient production has become an urgent problem. We have been trying to solve this problem via two approaches: the development of a more efficient conjugation method and the solid phase synthesis of globo-H. A solution to the conjugation problem, new method utilizing MMCCH linker, was developed to significantly improve the coupling of synthetic glycosides to the carrier protein. As for solid phase synthesis of globo-H, much insight has been gained on glycosidations on solid support, while the synthesis is still in progress. As a result, the trisaccharide intermediate of globo-H was successfully synthesized on the solid support, thus resulted in overall improvement in the synthetic efficiency.

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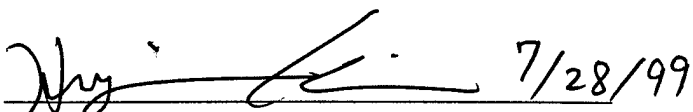

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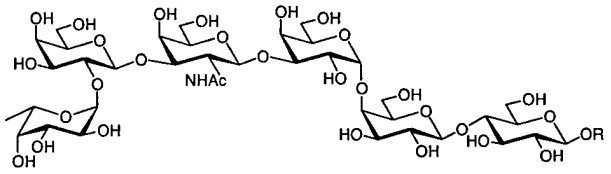
Introduction:

It is well known that carbohydrates play significant roles in intracellular and intercellular recognition.[1] Understanding these roles in the context of cancer immunology and developing cancer vaccines based on the immunological findings has been one of the major recent endeavors in the Danishefsky group.[2]

Many approaches toward the cancer vaccine development have been actively pursued with varied degrees of success. These include a neoglycoprotein approach that utilizes the conjugated carbohydrate antigens on immunogenic carrier proteins like KLH (keyhole limpet hemocyanin: Acronyms are listed on p 19.), and a mucin type peptide approach that employs clustered α-O linked synthetic glycopeptides. Various target antigens in these studies include Le^y, KH-1, N3 and globo-H.

As noted in the last annual report, globo-H (**1**) was shown to elicit humoral responses in mice,[3] and a new monoclonal antibody has been raised against it.[4] Furthermore, clinical studies were launched for patients with prostate cancer who have relapsed after primary therapies such as radiation or surgery. The administered vaccine, composed of globo-H-KLH conjugate and immunologic adjuvant QS-21, was found to be safe and capable of inducing specific high-titer IgM antibodies against globo-H.[5] As these clinical studies progress, it has become clear that a more efficient route to synthetic globo-H antigen is imperative to ameliorate the shortage of material.



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R = allyl : globo-H allylglycoside
R = ceramide : globo-H

As outlined in the original proposal, a more efficient production of globo-H can be achieved in three ways. One way is to improve the original solution synthesis of globo-H, while development of a new conjugation method of globo-H antigen to KLH carrier protein and a solid phase synthesis of globo-H constitute the second and the third way respectively. An improved solution phase synthesis has been developed recently, but will not be delineated in detail in this report since it has little to do with the original proposal and the statement of work. Objective 1, 2, and 3 in the statement of work was achieved by following the original synthetic procedures,[6] and sufficient amount of globo-H for the clinical studies mentioned above were obtained in the process. The glycoconjugation studies and solid phase synthesis of globo-H, which cover objective 4 and 5 in the statement of work, will be described in this report.

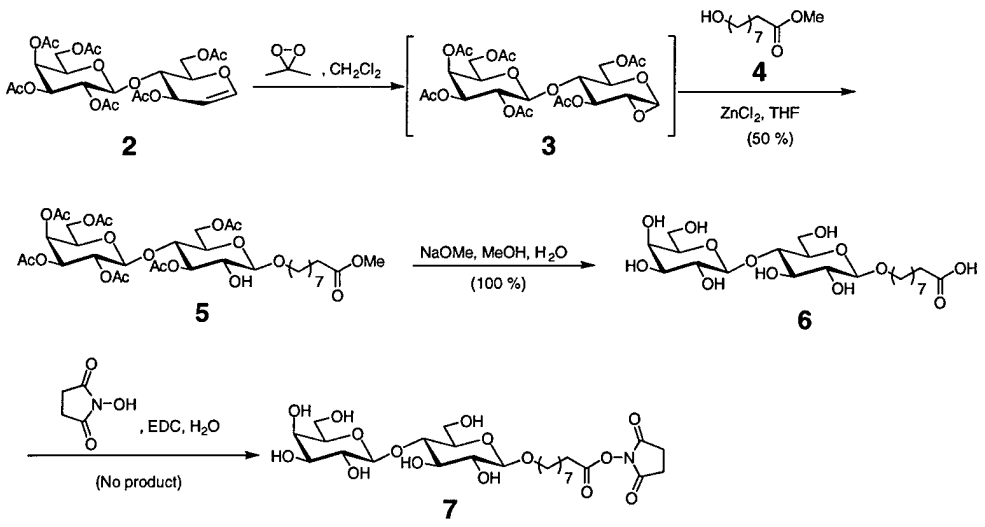
I. Conjugation Method Study (Objective 4)

As mentioned in the last annual report, a more efficient conjugation method of carbohydrate to the appropriate carrier protein was needed to improve the immunogenicity of the carbohydrate antigen. Keyhole lympet hemocyanin (KLH) protein was chosen to be a suitable carrier in these studies due to its better immunogenicity in mammals.[7] The conventional reductive amination method[8] was sufficient for the preliminary biological studies, however, the conjugation efficiency was unacceptably low and significant amounts of precious synthetic globo-H antigen were lost at this stage. It was clear that a method to improve this conjugation step was surely needed.

One of the first method devised involved N-hydroxy succinimide esters. Per-acetylated lactal **2** was chosen as a model in the conjugation reaction (Scheme 1), and a solution of **2** in methylene chloride was treated with dimethyldioxirane in acetone at 0 °C under N_2 atmosphere

for 30 minutes to yield the lactal epoxide **3**. Epoxide **3** was subsequently treated with a solution of hydroxy ester **4** in THF in the presence of zinc (II) chloride to give lactose ester **5** in 50 % yield. Acetyl groups of **5** were cleanly removed and the ester was saponified in one pot by treatment with sodium methoxide in methanolic water to give **6** in quantitative yield. Unfortunately, thought, the conversion of acid **6** to *N*-hydroxy succinimide ester **7** could not be achieved despite the numorous attempts.

Scheme 1 Conjugation Model Study with Lactal.



Parallel to these efforts, Dr. Govindaswami Ragupathi, *et. al.*, who were in close collaboration with us in this cancer vaccine program, carried out their own conjugation study, and they developed a method that significantly improved the conjugation efficiency.[9] The method utilized 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH) linker. Typically, the synthetic allyl glycoside **8** was ozonolized to yield the corresponding terminal aldehyde **9**, and it was reductively aminated with 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide in the presence of $NaBH_3CN$ to give **10** (Scheme 2). At the same time, KLH

was thiolated with 2-iminothiolane. Then, **10** was reacted with the thiolated KLH at pH 7.2 in 0.1 M sodium phosphate buffer overnight to furnish the glycoconjugated protein **11**. This procedure was repeated for various carbohydrate antigens and found to be superior to the previous direct reductive amination method in terms of both efficiency and the epitope ratio (Table 1).

Scheme 2 Conjugation Method Using MMCCH Linker

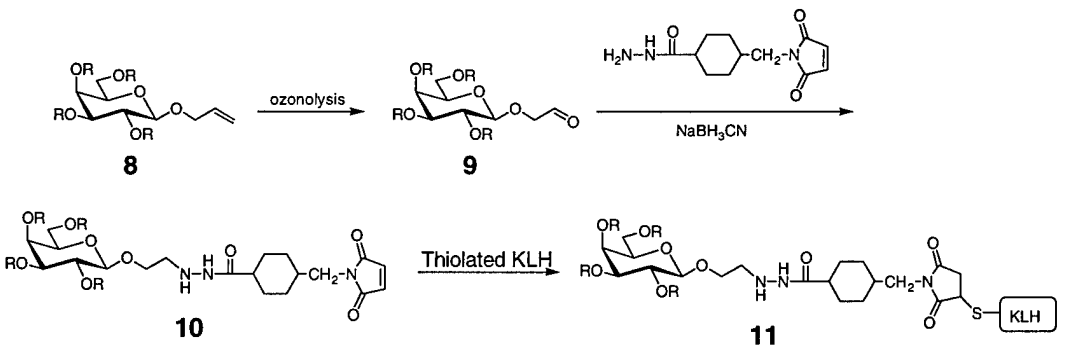


Table 1 : Comparison of EpitopeRatio on KLH by Conjugation Method*.

	direct reductive amination	MMCCH linker method
Le^y	848	1857
KH-1	141	492
globo-H	560	2365

* Number corresponds to the number of carbohydrates on a KLH protein on average. Roughly the same equivalents of carbohydrate per KLH protein were used for both methods.

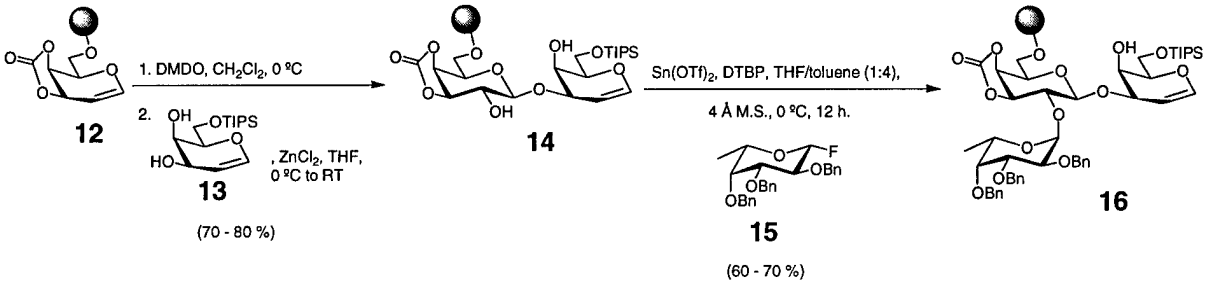
Thus, the study to improve the conjugation step was completed, and objective 4 in the statement of work was achieved with the new MMCCH linker method. At present this new linkage method is widely utilized in the conjugation effort.

II. Solid Phase Synthesis of Globo-H Hexasaccharide (Objective 5)

Solid phase synthesis of globo-H antigen was pursued as another way to improve the supply of this antigen. Many strategies for the solid phase carbohydrate synthesis have been developed.[10] Nevertheless, none of the strategies are general and efficient. In the Danishefsky group, the glycal based solid phase synthesis of oligosaccharides has been proven successful in many complex carbohydrate synthesis.[11] This strategy was again applied to the solid phase synthesis of globo-H.

The globo-H solid phase synthesis commenced with known solid phase bound galactal **12**[12] (Scheme 3). Solid phase bound **12** was suspended in anhydrous CH_2Cl_2 and treated with dimethyldioxirane at 0 °C. The resulting epoxide was treated with 6-TIPS galactal **13** in the presence of $ZnCl_2$ to provide disaccharide **14** on the solid support. The coupling efficiency was calculated by cleaving and isolating this disaccharide from the support by the treatment with TBAF, and it corresponded to greater than 70 % yield. Allyields given were obtained in a like fashion.

Scheme 3 Solid Phase Synthesis of Trisaccharide



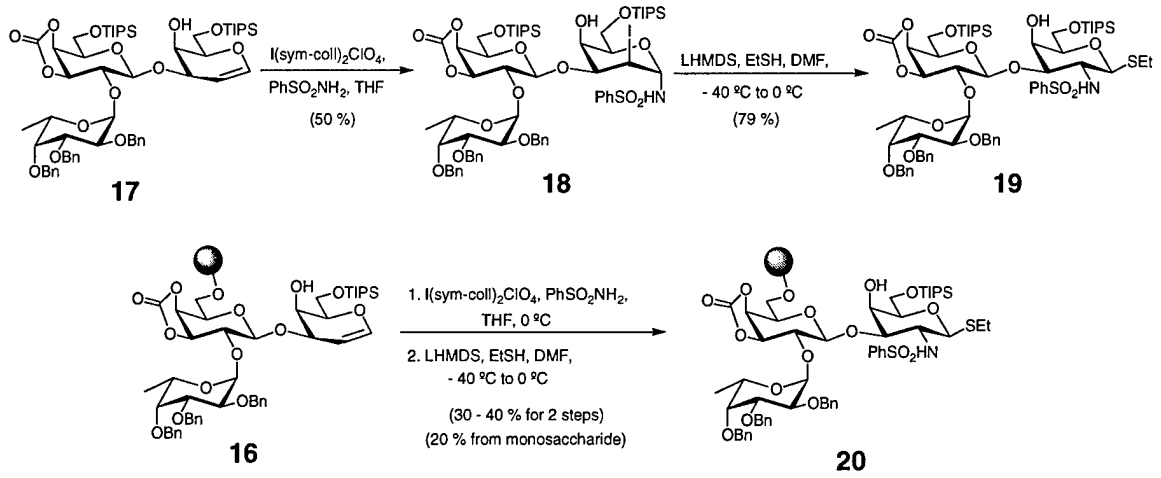
Fucosylation on **14** was tried with fluorofucosyl donor **15**. This reaction required many technical modifications. For example, it became apparent after many trials that the reaction

required the presence of dried 4 Å molecular sieves in the flask. However, it had been predicted to be difficult to separate solid molecular sieves from the solid support after the reaction. Ultimately, a separation method was devised that utilized the density differences. When suspended in anhydrous acetone, the molecular sieves were found to float on the surface of the solvent while the solid support stayed at the bottom of the reaction vessel. Thus, molecular sieves that floated on the surface could be removed via syringe.

Fortunately, the fucosylation reaction of **14** was very selective. In solution phase, similar reaction gave a mixture of two regioisomeric trisaccharides, which could not be separated easily, in a moderate yield (6:1 ratio in favor of the desired trisaccharide). However, on solid support, the desired trisaccharide **16** was obtained in a good yield with only trace amount of byproducts (60 – 70 % yield).

The next sequence, the installation of 2-amino-2-deoxy moiety, had been troublesome in the solution phase synthesis. Iodosulfonamide reaction on galactal **17** gave a mixture which contained the desired iodosulfonamide **18** (Scheme 4). However, the amount of **18** diminished as the time progressed, thus rendering the purification very difficult. For that reason, two step sequence of iodosulfonamidation and ethane thiolate mediated rearrangement to **19** was typically carried out without purification, and the yield for the sequence was typically low and inconsistant (around 40 – 50 % yield for the two steps). The same sequence on solid phase bound trisaccharide **16** was proven to be no better, and the solid phase bound trisaccharide thio-donor **20** was obtained in 30 – 40 % yield. Nevertheless, the presence of the thioglycoside was verified by cleaving and isolating it from the solid support.

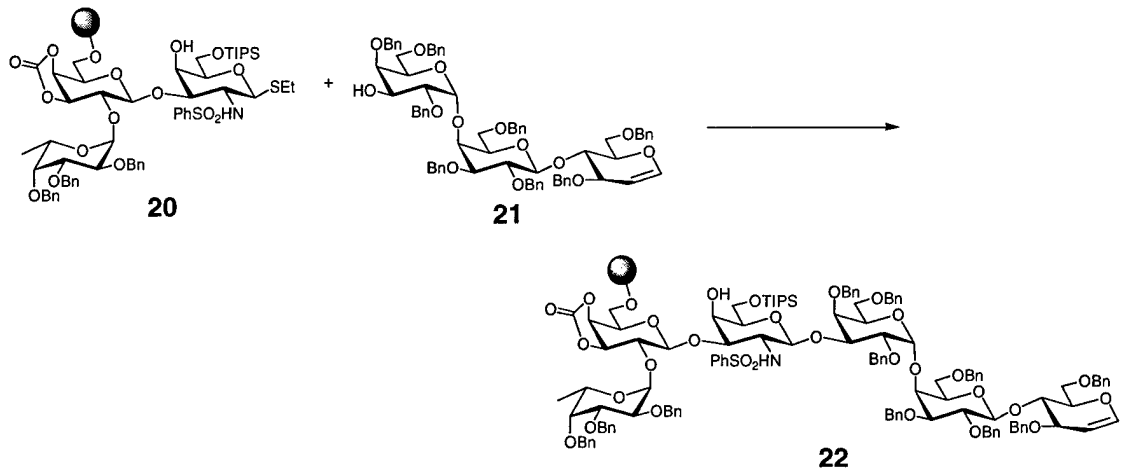
Scheme 4 Synthesis of Thioglycoside in Solid and Solution Phase



As with the solution phase synthesis, the most crucial stage of the solid support synthesis was the 3 + 3 coupling of **20** and **21**[6] (Scheme 5). Preliminary coupling study yielded the desired hexasaccharide **22**. However, subsequent effort to find optimal condition was fruitless. The results of the subsequent efforts are summarized in Scheme 5. In short, the reaction turned out to be capricious, and so far no optimized condition has been found. The reaction is dependent on the integrity of the thioglycoside **20**. At this point, there is no clear way to confirm the integrity of the thiodonor **20** over the two step sequence of iodosulfonamidation and thioethyl rearrangement. Work is in progress to solve this crucial problem.

Thus, study toward the solid supported synthesis of globo-H is still in progress in the Danishefsky group. As described above, task 8, 9, and 10 of the objective 5 was successfully completed.

Scheme 5 Attempted 3 + 3 Coupling on Solid Support.



MeOTf	di-*t*-butylpyridine	solvent	temperature	4 Å M.S.	Result
40 equiv.	40 equiv.	CH_2Cl_2/Et_2O	0 ºC (10 min.) to RT (5 h.)	not used	No product
20 equiv.	20 equiv.	same as above	same as above	not used	No product
10 equiv.	10 equiv.	same as above	same as above	not used	No product
10 equiv.	not used	same as above	0 ºC (5 h.)	not used	No product
20 equiv.	not used	CH_2Cl_2	0 ºC to RT (5 h.)	used	No product
20 equiv.	20 equiv.	CH_2Cl_2	0 ºC (10 h.)	used	No product
20 equiv.	20 equiv.	THF	0 ºC to RT (5 h.)	used	THF polymerized
20 equiv.	20 equiv.	CH_2Cl_2/Et_2O	0 ºC (10 h.)	used	No product

Experimental Section

General Methods. All chemicals used were reagent grade and used as supplied except where noted. THF was distilled from sodium/benzophenone ketyl under N_2. CH_2Cl_2 was distilled from calcium hydride under N_2. Analytical thin-layer chromatography was performed on E. Merck silicagel 60 F_{254} plates (0.25 mm). Infrared spectra were recorded on a Perkin Elmer 1600 series FT IR. ^1H NMR spectra were obtained on a Bruker AMX 400 (400 MHz) and DRX 500 (500 MHz) and are reported in parts per million (δ) relative to tetramethylsilane (0.00 ppm) or $CHCl_3$ (7.24 ppm). ^{13}C NMR spectra were obtained on a Bruker AMX 400 (100 MHz) and DRX 500 (125 MHz) and are reported in parts per million (δ) relative to $CDCl_3$ (77.0 ppm) as an internal reference.

Conjugation Method Using MMCCH Linker. For detailed procedure, consult Govindaswami Ragupathi et. al. *Glycoconjugate J.* **15**, 217 (**1998**). Below is the exemplary procedure for the conjugation of KH-1 to KLH using MMCCH linker.

Preparation of KH-1-MMCCH. To a solution of 2 mg of KH-1 aldehyde in 1 mL of 0.1 M sodium acetate buffer pH 5.5 was added a solution of 4 mg of MMCCH in 100 μL of dimethyl sulfoxide. The reaction mixture was incubated at RT for 15 min with gentle stirring. At the end of 15 min, 2 mg of sodium cyanoborohydride was added and the incubation continued at RT for 2 h. Unreacted MMCCH was removed in a Sephadex G10 column equilibrated previously with 0.1 M sodium phosphate buffer pH 6.0 containing 5 mM EDTA and eluted with the same buffer. The fractions positive for KH-1 by TLC with orcinol were combined.

Addition of sulfhydryl groups to KLH. To 4 mg of KLH was added a solution of 2-iminothiolane (2 mg) in thiolation buffer (50 mM triethanolamine, 0.15 M NaCl, 5 mM EDTA, pH 8.0), and the mixture was incubated with stirring at RT for 2 h. Unreacted 2-iminothiolane was removed by Sephadex G15 column equilibrated previously with 0.1 M sodium phosphate buffer pH 7.2 containing 5 mM EDTA and eluted with the same buffer. Fractions positive for KLH with BioRad protein assay dye reagent were combined.

Conjugation of KH-1-MMCCH to thiolated KLH. The KH-1-MMCCH product and thiolated KLH were mixed and adjusted to pH 7.2 with 0.1 M sodium phosphate buffer pH 8.0. The reaction mixture was then incubated at RT overnight. The content of the KH-1-MMCCH-KLH reaction vial was transferred to a Centriprep concentrator 30 (Amicon: molecular cut-off 30000 Dalton) and unreacted KH-1-MMCCH ws removed completely with multiple washes.

General Precedure for Retrieval of Glycals from the Solid Support. Polymer-bound glycal 12 (60 mg) was suspended in 1.5 mL of THF, and the suspension was treated with 0.2 mL of 1.0

M AcOH in THF and 0.4 mL of 1.0 M TBAF in THF. The mixture was stirred at 40 °C for 18 h, and the polymer was washed with CH_2Cl_2 (2 x 5 mL) and THF (2 x 5 mL). The combined washes were concentrated and purified by column chromatography on silica gel (30:1 Et_2O:MeOH) to give glycal as a colorless gum (5.5 mg, 32 μmol). The loading of this batch of polymer-bound glycal was determined to be 0.53 mmol/g of glycal per gram of resin.

Synthesis of Polymer-bound Disaccharide 14. Polymer-bound galactal **12** (1.67g, 0.53 mmol/g, 0.8851 mmol) was placed in a 100 mL solid-phase synthesis flask and suspended in 20 mL of anhydrous CH_2Cl_2 under N_2. The suspension was cooled to 0 °C and treated with 50 mL of dimethyldioxirane solution (ca. 0.1 M in acetone). The mixture was stirred at 0 °C for 90 min and the liquid was removed by filteration with positive N_2 pressure. The polymer was resuspended in 20 mL of CH_2Cl_2 uner N2, treated once more at 0 °C with 50 mL of dimethyldioxirane solution, stirred for 40 min, filtered, and dried in vacuo. To the polymer-bound epoxide under N_2 was added a solution of **13** (2.0 g, 6.61 mmol) in anhydrous THF (10 mL). The suspension was cooled to 0 °C, treated with 1.0 mL of 1.0 M $ZnCl_2$ in ether, and stirred for 8h while allowed to slowly warm to RT. Then, the polymer was rinsed with THF (4 x 20 mL) and dried in vacuo to give 1.9 g compound **14** (0.7087 mmol, 80 % yield) as a colorless powder. Polymer **14** (105 mg) gave 13.1 mg glycal (13.1 mg, 39.21 μmol) upon cleavage with TBAF.

$[\alpha]^{23}_D$ = - 42.81° (*c* 0.64, acetone); IR (thin film) 3370, 2927, 1797, 1648, 1374, 1235, 1167, 1069 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ 6.34 (dd, 1H, *J* = 1.7, 6.3 Hz), 5.01 (dd, 1H, *J* = 1.9, 7.3 Hz), 4.84 (dd, 1H, *J* = 5.9, 7.3 Hz), 4.78 (d, 1H, *J* = 6.7 Hz), 4.67 (m, 1H), 4.55 (m, 1H), 4.13 – 4.11 (m, 2H), 3.90 (m, 1H), 3.79 – 3.75 (m, 5H), 3.67 (t, 1H, *J* = 6.1 Hz); ^{13}C NMR (125

MHz, acetone-d_6) δ 155.0, 145.6, 100.9, 99.6, 79.6, 77.9, 75.7, 73.2, 73.1, 72.0, 64.9, 61.7, 61.2; HRMS (FAB) calcd. For $C_{13}O_{10}H_{18}Na$: 357.0797, found 357.0789.

Synthesis of Polymer-bound Trisaccharide 16. To a mixture of 1.147 g of polymer-bound disaccharide **14** (0.38 mmol/g, 0.436 mmol) and 2.1g activated 4 Å M.S. were added 2.1 g of tribenzyl fucosylfluoride (**15**) (4.811 mmol) in THF (5 mL) via cannula. The suspension was diluted with 40 mL toluene. To the mixture was added 1.9 mL of di-*tert*-butylpyridine (8.66 mmol) and the suspension was stirred at RT for 20 min. The suspension was cooled to 0 °C, and was treated with 1.8 g of Sn(OTf)2 (4.33 mmol) in 5 mL of THF via cannula. The suspension was stirred for 8 h while allowed to warm to RT. The mixture was filtered and resuspended in acetone to allow for the removal of the molecular sieves. The polymer was further washed with acetone, DMSO, CH_2Cl_2, and THF. The polymer was dried in vacuo overnight to provide 1.9 g of **16** (0.310 mmol, 70 % yield). Polymer-bound trisaccharide **16** (50 mg) provided glycal (8.2 mg, 10.93 μmol).

$[\alpha]^{22}_D$ = - 96.0° (*c* 0.9, CH_3OH); FTIR (thin film) 3440, 2929, 1804, 1650, 1496, 1454, 1362, 1243, 1081, 1047, 739, 698 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 7.44 – 7.29 (m, 15H), 6.30 (dd, 1H, *J* = 1.7, 6.3 Hz), 5.24 (d, 1H, *J* = 3.4 Hz), 5.04 – 4.98 (m, 3H), 4.96 (d, 1H, *J* = 5.9 Hz), 4.83 – 4.80 (m, 4H), 4.65 (d, 1H, *J* = 11.3 Hz), 4.59 (m, 1H), 4.51 (m, 1H), 4.26 – 4.20 (m, 1H), 4.20 – 4.10 (m, 3H), 4.50 – 3.97 (m, 2H), 3.90 – 3.70 (m, 9H), 3.24 (m, 1H), 1.22 –1.20 (d. 3H, *J* = 7.2 Hz); ^{13}C (100 MHz, acetone-d6) δ 155.3, 146.1, 140.7, 140.6, 140.5, 129.5, 129.3, 129.0, 128.7, 128.6, 128.5, 100.4, 100.2, 98.3, 80.0, 79.5, 79.0, 78.6, 77.4, 76.1, 75.9, 75.8, 74.3, 73.9, 73.5, 68.4, 65.4, 62.4, 61.8; HRMS (FAB) calcd. for $C_{40}H_{46}O_{16}Na$: 773.2886, found 773.2758.

Synthesis of Polymer Bound Thioglycoside 20. To the mixture of 269 mg of polymer-bound trisaccharide **16** were added 140 mg of benzene sulfonamide (0.89 mmol, 5.5 eq.) in 20 mL

anhydrous THF. Then the suspension was treated with 605 mg of I(*sym*-coll)$_2$ClO$_4$ (1.29 mmol, 8 eq.) at 0 °C. The mixture was stirred at 0 °C for 2 h and quenched with sat. $Na_2S_2O_3$ solution. The polymer was filtered, washed with water, acetone, DMSO, acetone, CH_2Cl_2, and THF, and dried in vacuo. The iodosulfonamide intermediate was suspended in 10 mL of anhydrous DMF and cooled to – 40 °C. Then 0.24 mL of ethanethiol (3.2 mmol, 20 eq.) and 1.3 mL of 1.0 M solution of LHMDS (1.3 mmol, 8 eq.) in THF were added in sequence. The suspension was gradually warmed to 0 °C over 2 h and stirred for additional 4 h. The reaction was quenched with sat. NH_4Cl, filtered, and washed with water, acetone, DMSO, acetone, and THF. The polymer was dried in vacuo to yield 265 mg of polymer-bound thioglycoside **20** (20 % overall yield from **12**). Compound 20 (70 mg) provided 5 mg of thioglycoside (5.17 μmol) upon cleavage with TBAF.

$[\alpha]^{22}_D$ = - 92.36 ° (*c* 0.55, CH_2Cl_2); FTIR (thin film) 3488, 2926, 1800, 1451, 1325, 1160, 1092, 1050 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, 2H, J = 5.6 Hz), 7.48 – 7.40 (m, 4H), 7.39 – 7.26 (m, 14H), 5.59 (d, 1H, J = 6.9 Hz), 5.20 (d, 1H, J = 3.3 Hz), 4.99 – 4.95 (m, 2H), 4.86 (d, 1H, J = 11.4 Hz), 4.73 – 4.55 (m, 8H), 4.11 – 3.53 (m, 20H), 2.96 (b, 1H), 2.55 – 2.37 (m, 2H), 1.78 (b, 1H), 1.18 (d, 3H, J = 6.4 Hz), 1.10 (t, 3H, J = 7.4 Hz); 13C NMR (100 MHz, CDCl3) δ 155.1, 141.9, 138.6, 138.5, 138.4, 132.5, 128.8, 128.6, 128.3, 128.2, 128.0, 127.6, 127.5, 127.4, 127.2, 99.3, 98.0, 84.3, 80.5, 78.8, 77.9, 77.3, 76.0, 75.0, 74.5, 74.0, 73.4, 72.4, 69.9, 68.4, 67.8, 62.1, 62.0, 55.1, 24.0, 16.8, 14.6; HRMS (FAB) calcd. for $C_{48}H_{57}O_{16}NS_2Na$: 990.3119, found 990.2978.

Conclusion

Two ways to improve the supply of globo-H antigen for clinical studies, namely the solid support synthesis of globo-H and the development of a new conjugation method, have been

pursued. Conjugation using a new linker, MMCCH, was ultimately identified as the optimal method. For the solid phase synthesis of globo-H, the optimal condition for fucosylation was found, and trisaccharide was successfully built on the solid support in high efficiency (50 - 60 % overall yield compared with 40 % in solution phase synthesis). In addition, a method to use molecular sieves in the solid phase reaction was devised and successfully applied in the fucosylation reaction. Thus, the full benefits of solid phase synthesis were realized even at this stage. More efficient route to the trisaccharide **16** was realized by eliminating the need for purification and isolation of intermediates, thus improving overall synthesis of globo-H. The efforts toward the solid phase synthesis of globo-H is in progress.

Reference:

1. T. W. Rademacher, R. B. Parekh, R. A. Dwek, *Ann. Rev. Biochem.* **57**, 785 (1988); A. Varki, *Glycobiology* **3**, 97 (1993).

2. S. J. Danishefsky, J. R. Allen, *Angew. Chem. Int. Ed.* Submitted.

3. G. Ragupathi, T. K. Park, S Zhang, I. J. Kim, L. Graber, S. Adluri, K. O. Lloyd, S. J. Danishefsky, P. O. Livingston, *Angew. Chem. Int. Ed. Engl.* **36**, 125 (1997).

4. V. Kudryashov, G. Ragupathi, I. J. Kim, M. E. Breimer, S. J. Danishefsky, P. O. Livingston, K. O. Lloyd, *Glycoconjugate J.* **15**, 243 (1998).

5. G. Ragupathi, S. F. Slovin, S. Adluri, D. Sames, I. J. Kim, H. M. Kim, M. Spassova, W. G. Bornmann, K. O. Lloyd, H. I. Scher, P. O. Livingston, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **38**, 563 (1999); S. F. Slovin, G. Ragupathi, S. Adluri, G. Ungers, K. Terry, S. Kim, M. Spassova, W. G. Bornmann, M. Fazzari, L. Dantis, K. Olkiewicz, K. O. Lloyd, P. O. Livingston, S. J. Danishefsky, H. I. Scher, *Proc. Natl. Acad. Sci. USA* **96**, 5710 (1999).

6. T. K. Park, I. J. Kim, S. Hu, M. T. Bilodeau, J. T. Randolph, O. Kwon, S. J. Danishefsky, *J. Am. Chem. Soc.* **118**, 11488 (1996).

7. F. Helling, Y. Shang, M. Calves, H. F. Oettgen, P. O. Livingston, *Cancer Res.* **54**, 197 (1994).

8. M. A. Bernstein, L. D. Hall, *Carbohydr. Res.* **78**, C1 (1980).

9. G. Ragupathi, R. R. Koganty, D. Qiu, K. O. Lloyd, P. O. Livingston, *Glycoconjugate J.* **15**, 217 (1998).

10. J. M. J. Fréchet, in *Polymer-Supported Synthesis of Oligosaccharides*, P. Hodge, D. C. Sherrington, Eds. (Wiley, Chichester, 1980); R. Rodebaugh, S. Joshi, B. Fraser-Reid, M. H. Geysen, G. M. Paul, *J. Org. Chem.* **62**, 5660 (1997); J. Rademann, R. R. Schmidt, *ibid.* **62**, 3650 (1997); I. Yakishige, T. Ogawa, *J. Am. Chem. Soc.* **119**, 5562 (1997); K. C. Nicolaou, N. Watanabe, J. Li, J. Pastor, N. Winssinger, *Angew. Chem. Int. Ed.* **37**, 1559 (1998); R. Liang, L. Yan, J. Loebach, M. Ge, Y. Uozumi, K. Sekanina, N. Horan, J. Gildersleeve, C. Thompson, A. Smith, K. Biswas, W. C. Still, D. Kahne, *Science* **274**, 1520 (1996).

11. P. H. Seeberger, S. J. Danishefsky, *Acc. Chem. Res.* **31**, 685 (1998).

12. J. T. Randolph, K. F. McClure, S. J. Danishefsky, *J. Am. Chem. Soc.* **117**, 5712 (1995)

Acronyms:

THF : tetrahydrofuran
Ac : acetyl
TIPS : triisopropylsilyl
DMDO : dimethyldioxirane
DTBP : di-tert-butylpyridine
Bn : benzyl
LHMDS : lithium bis(N,N-dimethyl)amide
Ph : phenyl
EtSH : ethanethiol
DMF : dimethylformamide

Key Research Accomplishments:

- Solution phase synthesis of globo-H for the immunological and clinical studies.
- More efficient conjugation of globo-H to KLH using MMCCH linker.
- Development of a method to use solid molecular sieves in solid phase synthesis.
- Efficient solid phase synthesis of trisaccharide intermediate for the globo-H.

Reportable Outcomes:

- P. P. Deshpande, H. M. Kim, A. Zatorski, T. –K. Park, G. Ragupathi, P. O. Livingston, D. Live, S. J. Danishefsky, "Strategy in Oligosaccharide Synthesis: An Application to a Concise Total Synthesis of the KH-1 (adenocarcinoma) Antigen," *J. Am. Chem. Soc.* **120**, 1600 (1998).
- G. Ragupathi, S. F. Slovin, S. Adluri, D. Sames, I. J. Kim, H. M. Kim, M. Spassova, W. G. Bornmann, K. O. Lloyd, H. I. Scher, P. O. Livingston, S. J. Danishefsky, "A Fully Synthetic Globo H Carbohydrate Vaccine Induces a Focused Humoral Response in Prostate Cancer Patients: A Proof of Principle," *Angew. Chem. Int. Ed.* **38**, 563 (1999).

Graduate Degree: Ph.D. in Chemistry will be awarded to Hyunjin M. Kim from Columbia University in October, 1999.

List of Personnel Received Pay from the Fellowship:

Hyunjin M. Kim.

J. Am. Chem. Soc. **1998,** *120,* 1600−1614

Strategy in Oligosaccharide Synthesis: An Application to a Concise Total Synthesis of the KH-1(adenocarcinoma) Antigen

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Abstract: A concise and potentially practical synthesis of the title compound has been achieved. The route features a high degree of convergence and economy of synthetic operations. A key step is the concurrent introductory addition of three α-L-fucosyl residues at required hydroxyl acceptor sites (see **37** → **39**). Conjugation to carrier protein was achieved, and a route to include truncated structures for investigations for antibody specificity was accomplished.

Background

Many tumors are characterized by the appearance of large and unusual oligosaccharide subtypes.[1,2] These structures tend to emerge covalently bound to proteins in the form of cell surface glycoproteins. Alternatively, the anomalous carbohydrates can be encountered as glycolipids, adhering to cell surfaces through attractive molecular forces rather than via classical covalent bonds. The isolation, immunocharacterization, and structural identification of large carbohydrate-based tumor antigens constitutes a highly complex and challenging undertaking.

The possibility of exploiting these tumor-associated carbohydrate ensembles to provoke productive immune responses to the transformed state has occurred to glycobiologists, immunologists, and clinicians for some time.[3,4] Progress along these lines had to await breakthroughs in isolation, and purification techniques, as well as in the ability to assign connectivity and stereochemistry of carbohydrate domains through spectroscopic methods. Fortunately, major advances in these areas have provided a series of interesting structures (proposed with varying degrees of rigor) that might form the basis of strategies to achieve active immunity. However, a complicating feature, not easily overcome, is the difficulty associated with isolating small amounts of elaborate glycoconjugate structures from tissue collections of patients. Moreover, retrieval of the intact carbohydrate lattice by severing its covalent attachment to bioconjugating molecules (proteins or lipids) is only rarely possible.

These complexities pose large challenges as well as important opportunities for organic synthesis. There is, first, a great need for synthesizing the epitope structures themselves. However, superimposed on this goal is the challenge of delivering the tumor antigens in a favorable molecular framework for eliciting therapeutically useful, active immunity. The ultimate success of the chemistry end of the enterprise, then, is measured not only by the attainment of the total synthesis of the epitope sector, but also by the incorporation of this epitope into carrier protein. The total construct becomes the subject of immunological evaluation as part of a coherent vaccinology program.

The strategy and methodology which we have been employing for synthesizing the oligosaccharide epitopes are conveniently grouped under the term "glycal assembly".[5] The logic of the method has been amply reviewed. The employment of glycal assembly in the construction of epitope structures including the follow-up bioconjugation and mouse immunization studies have resulted in a vaccine (Globo-H)[6,7] which is currently undergoing advanced clinical evaluation. Two other fully synthetically derived vaccines are at the stage of advanced, preclinical processing.

In this paper, we deal with what is perhaps the most formidable carbohydrate-based tumor antigen thus far character-

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[∥] Current address: The Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

(1) Hakomori. S. *Cancer Res.* **1985**, *45*, 2405. Feizi. T. *Cancer Surveys* **1985**, *4*, 245; Lloyd, K. O. *Am. J. Clin. Pathol.* **1987**, *87*, 129. Lloyd, K. O. *Cancer Biol.* **1991**, *2*, 421.

(2) (a) Hakomori, S. *Cancer Cells* **1991**, *3*, 461. (b) Hakomori, S. *Adv. Cancer Res.* **1989**, *52*, 257.

(3) Toyokuni, T.; Singhal, A. K. *Chem. Soc. Rev.* **1995**, *24*, 231.

(4) Hakomori, S.; Zhang, Y. *Chem. Biol.* **1997**, *4*, 97.

(5) Bilodeau, M. T.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1380.

(6) (a) Park, T.-K.; Kim, I. J.; Hu, S.; Bilodeau, M. T.; Randolph, J. T.; Kwon, O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1996**, *118*, 11488. (b) Bildoeau, M. T.; Park, T.-K.; Hu, S.; Randolph, J. T.; Danishefsky, S. J.; Livingston, P. O.; Zhang, S. *J. Am. Chem. Soc.* **1995**, *117*, 7840. (c) Kim, I. J.; Park, T.-K.; Hu, S.; Abrampah, K.; Zeng, S.; Livingston, P. O.; Danishefsky, S. J. *J. Org. Chem.* **1995**, *60*, 7716.

(7) Ragupathi, G.; Park, T.-K.; Zhang, S.; Kim, I. J.; Graber, L.; Adluri, S.; Lloyd, K. O.; Danishefsky, S. J.; Livingston, P. O. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 125.

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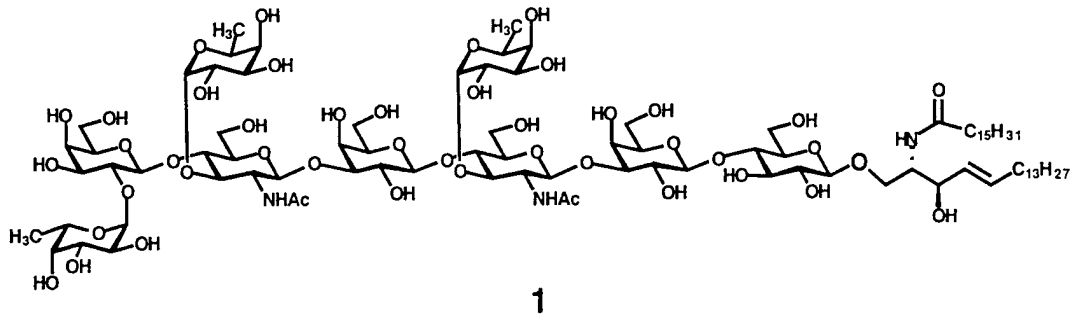

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Figure 1.

ized. The KH-1 antigen (**1**; see Figure 1)[8] was isolated from human colonic adenocarcinoma cells by using antibodies generated against the classical Le^y determinant. System **1**, in the form of glycolipid conjugates, was found to be present on the cell surface of all adenocarcinoma cells thus far studied. Furthermore, its presence has never been detected in normal colonic extracts.

Monoclonal antibodies[9a] were raised against this antigen and found to bind specifically to compound **1**. On the basis of these studies, Hakomori et al.[9b] postulated that the KH-1 antigen is a highly specific marker for malignancy and premalignancy involving colonic adenocarcinoma. Recently, an X-ray crystal structure of an antitumor antibody BR96 in complex with the nonanoate ester derivative of Le^y tetrasaccharide was reported by Jeffrey et al.[10] The view of the antibody−Le^y complex provided by this determination suggested that the BR96 antibody has unused binding capacity which might also recognize structures larger than the Le^y tetrasaccharide (such as the KH-1 antigen).

The difficulties associated with isolation and separation of complex carbohydrates from human colonic cancer tissue have been such that compound **1**, either as a glycolipid or as a protein conjugate, has not been available for evaluation. Thus, chemical synthesis could offer a viable alternative to produce workable quantities of complex systems such as the KH-1 antigen (**1**). Our interests were not limited to KH-1 (**1**), but included congeners[11] which would be bioconjugated to the appropriate protein carrier systems. Below, we describe (vide infra) the total synthesis of the KH-1 antigen **1**.[12] We also describe a protocol for reaching a suitable bioconjugatable analogue, as well as a truncated heptasaccharide congener. In addition, we also relate the upgrading of the KH-1 epitope for conjugation to carrier protein.

Synthetic Planning

In conducting this project, we also hoped to address the important issue of "strategy" in oligosaccharide synthesis. Of course, in this field, as opposed to "conventional" natural product synthesis, the basic building blocks are rather restricted and tend to bear obvious homology with the readily recognized components of the target system. Nonetheless, there are considerable opportunities for the realization of formats, which might lead to major synthetic economies. Thus, strategy in oligosaccharide synthesis tends to focus on programs for optimal conciseness in identifying specific centers for glycosylation, and for the attainment of stereocontrol in these couplings. *A cardinal strategic objective in oligosaccharide assembly is that of gaining maximum relief from blocking group manipulations.* From these perspectives, we came to favor a plan that would build a hexasaccharide (cf. structure **4**), so differentiated in terms of its protecting patterns as to allow for the simultaneous unveiling of the three free hydroxyls destined for fucosylation at a strategic point of our choosing. The three fucosylations would then be conducted concurrently.

Broadly speaking, our plan called for inclusion of three kinds of blocking groups in structure **4** (see Figure 2). The two nitrogen centers carry special R functions. The three oxygen centers to be fucosylated carry unique blocking groups (R*). The remaining hydroxyls are protected by a general blocking group (P). The three unique protecting groups would be cleaved in a single operation (**4** → **3**). Hopefully, the three immunologically defining α-L-fucose entities could be introduced in one concurrent synthetic operation (see structure **2**). The terminal glycal in **2** would be used to provide access to the native KH-1 antigen (**1**) or to bioconjugates, en route to evaluatable antiadenocarcinoma vaccines.

Considerable thought was also directed to assembling the hexasaccharide with minimal protection−deprotection maneuvers which would still be consistent with sound management of the complex network of hydroxyl groups. Toward this end, we found advantages in drawing from principles which have come forth from the logic of glycal assembly.[5] Thus, differentiated glycal types **6** and **7** (see Figure 3) were to be derived from D-glucal by exploiting reliable reactivity preferences of the C6, C3, and C4 hydroxyls (C6 > C3 > C4).[13] Moreover, the clean fashioning of an α-epoxide from appropriate galactal derivatives (cf. **5**) is well-known. Also well-known is the excellent β-galactosyl-donating capacity of properly chosen epoxides.[14]

Coupling of such a galactal-derived epoxide (**5**) to acceptors **6** and **7** would give rise to lactal-related disaccharides **8** and **9**, respectively. The C2′ hydroxyl of the lactal derivative **8** could be uniquely protected with R* in anticipation of future fucosylation at this site (see **10** bearing two unique R* blocking groups). In another arm of the effort, the same hydroxyl groups of **8** could be protected with a standard blocking (P) group to

(8) Nudelman, E.; Levery, S. B.; Kaizu, T.; Hakomori, S.-I. *J. Biol. Chem.* **1986**, *261*, 11247.

(9) (a) Kaizu, T.; Levery, S. B.; Nudelman, E.; Stenkamp, R. E.; Hakomori, S.-I. *J. Biol. Chem.* **1986**, *261*, 11254. (b) Kim, S. Y.; Yuan, M.; Itzkowitz, S. H.; Sun, Q.; Kaizu, T.; Palekar, A.; Trump, B. F.; Hakamori, S.-I. *Cancer Res.* **1986**, *46*, 5985.

(10) Jeffery, P. D.; Bajorath, J.; Chang, C. Y.; Dale, Y.; Hellstrom, I.; Hellstrom, E. K.; Sheriff, S. *Nat. Struct. Biol.* **1995**, *2*, 456.

(11) (a) Helland, A.-C.; Nilsson, M.; Norberg, T. *J. Carbohdyr. Chem.* **1992**, *11*, 77. (b) Windmuller, R.; Schimidt, R. R. *Tetrahedron Lett.* **1994**, *35*, 7927.

(12) (a) For a communication of parts of this work see: Deshpande, P. P.; Danishefsky, S. J. *Nature* **1997**, *387*, 164. (b) Another synthesis of nonconjugated KH-1 antigen was reported while our manuscript (ref 12a) was being reviewed: Hummel, G.; Schmidt, R. R. *Tetrahedron Lett.* **1997**, *38*, 1173.

(13) (a) Danishefsky, S. J.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Koseki, K.; Griffith, D. A.; Oriyama, T.; Marsden, S. P. *J. Am. Chem. Soc.* **1995**, *117*, 1940. (b) Danishefsky, S. J.; Behar, V.; Randolph, J. T.; Lloyd, K. O. *J. Am. Chem. Soc.* **1995**, *117*, 5701.

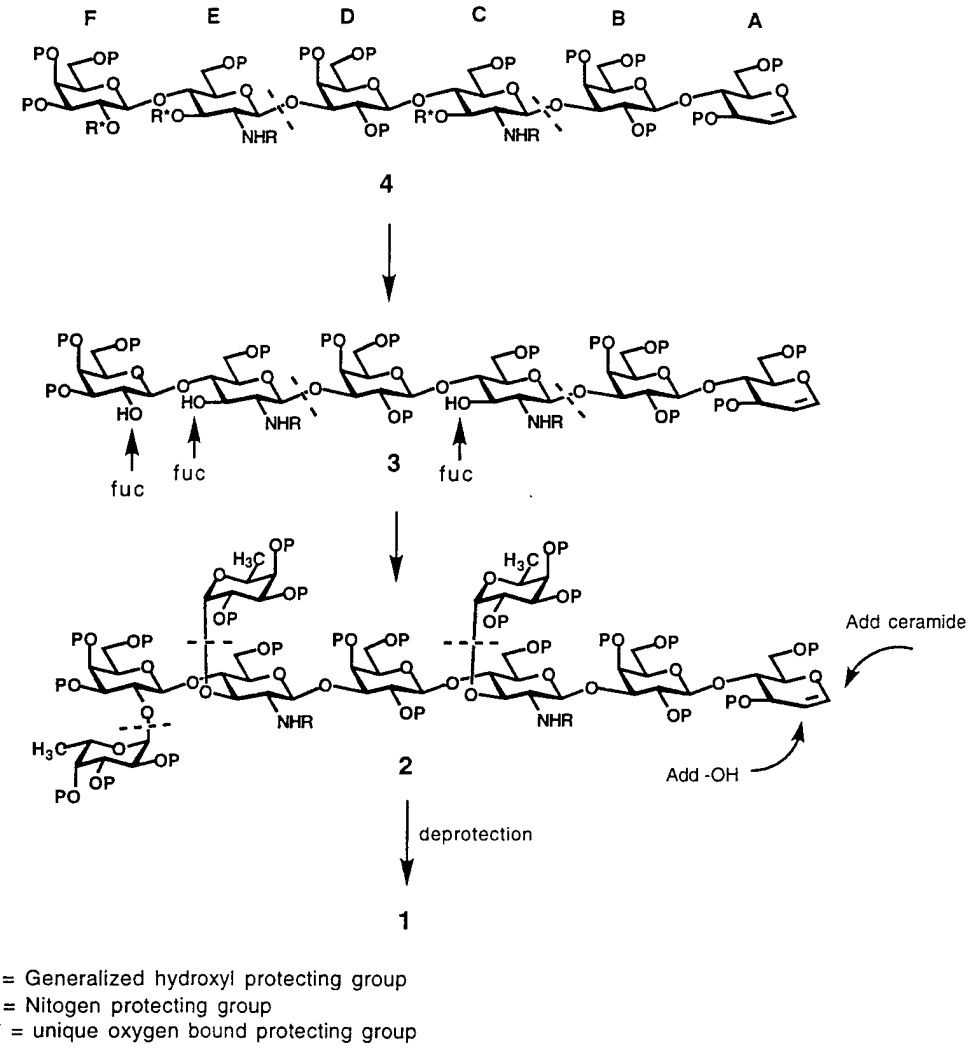


Figure 2.

afford **11**. Thus, segment **11** bears one unique blocking group (R*) anticipating fucosylation amidst more robust blocking groups (P). In some fashion (vide infra), lactal derivative **9** would be so arranged as to function as a glycosyl acceptor at C3′ (see system **12**).

Azaglycosylative coupling[6b,15] of **12** with **11** would provide tetrasaccharide **13**. In a manner to be discussed, the C3 hydroxyl of the remote galactose of **13** would be identified as an azaglycosyl acceptor site (see **14**). Coupling of **14** at this site with an azaglycosyl donor derived from **10** would afford **4**, thus feeding back in to the prospectus adumbrated in Figure 2.

Needless to say, considerable trial and error was to be necessary before translating this program to the realm of practice. An account of the manner in which implementation was accomplished in a highly concise fashion is described below.

Discussion and Results

The specific galactal derivative corresponding to formal structure **5** was the epoxide **17**. This compound was readily and stereospecifically obtained by action of dimethyldioxirane[16] on **16** (see Scheme 1). Compound **16** was synthesized through benzylation of the previously reported **15**.[17] As has been demonstrated many times,[5] the cyclic carbonate blocking group, engaging C3 and C4 of a 6-monoprotected galactal, has a very powerful α-directing effect in the epoxidation reaction, and a strong β-directing effect in the use of such an epoxide for galactosylation. These findings proved to be applicable to this synthesis (vide infra).

The specific glucals corresponding to formal structures **6** and **7** were **18** and **20**, respectively. The dibenzyl derivative **18** had been reported from our laboratory by controlled dibenzylation of D-glucal.[13b] The preparation of **20** involved a readily achieved mono-triethylsilylation of **19**. Compound **19** had been reported in the literature in very low yield by the monobenzylation of D-glucal at C6.[18] An improved but still unoptimized preparation of **19** was accomplished as part of our studies. With the required monosaccharide structures well in hand, glycal assembly could commence. Thus, coupling of **17** with **20** occurred under mediation by zinc chloride, affording **21** in 65% yield.

The lone hydroxyl group at C2′ of lactal product **21** was protected as its acetate (see compound **22**). In terms of our overall logic, this acetyl group serves as a general protecting

(14) Gervay, J.; Peterson, J. M.; Oriyama, T.; Danishefsky, S. J. *J. Org. Chem.* **1993**, *58*, 5465.
(15) Griffith, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 5811.
(16) Murray, R. W.; Jeyaraman, R. J. *J. Org. Chem.*, **1985**, *50*, 2847.
(17) Randolph, J. T.; McClure, K. F.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 5712.
(18) (a) Blackburne, I. D.; Burlitt, A. I. R.; Fredericks, P. F.; Guthrie, R. D. *Aust. J. Chem.* **1976**, *29*, 381. (b) Fischer, S.; Hamann, C. H. *J. Carbohydr. Chem.* **1995**, *14* (3), 327.

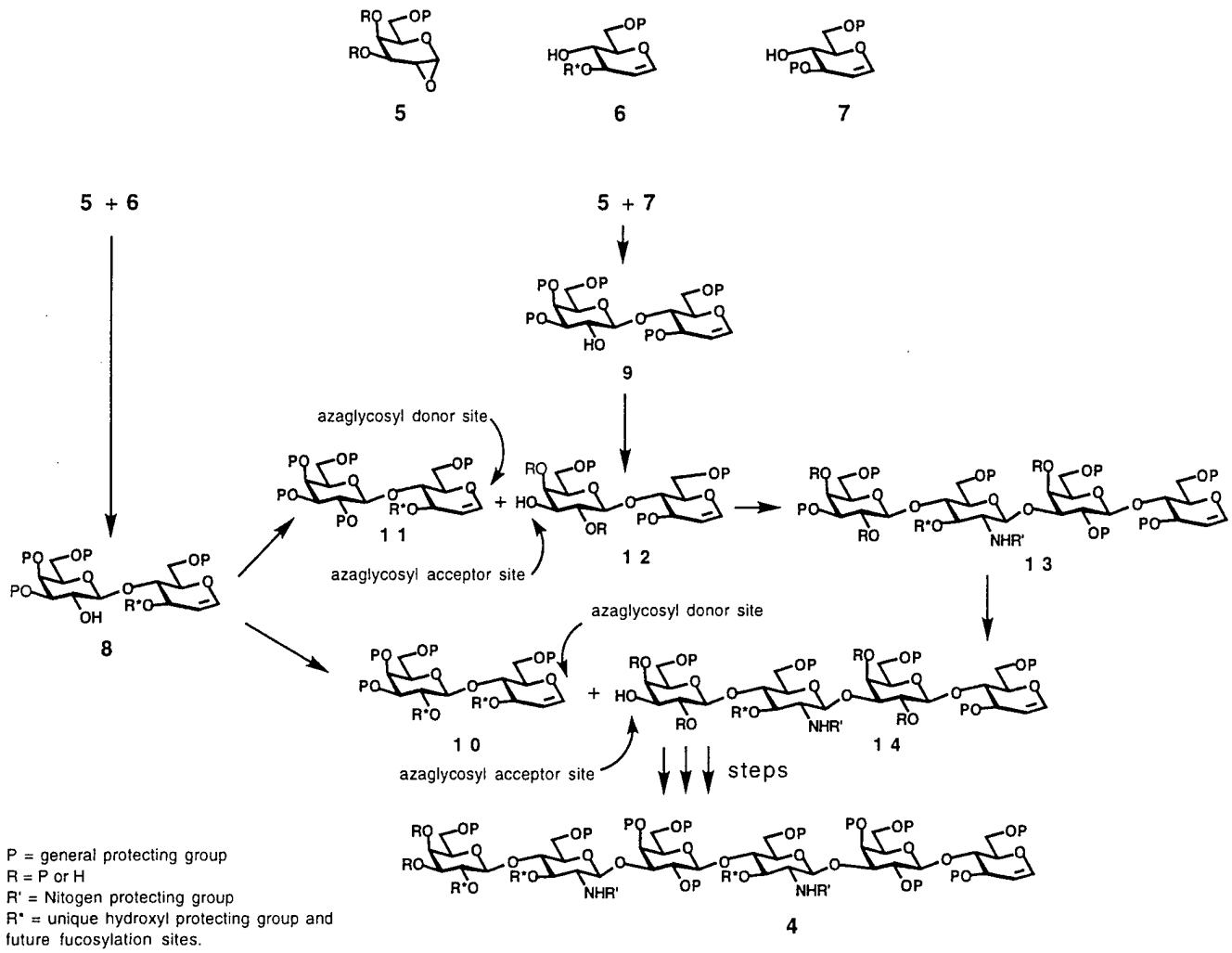


Figure 3.

group, P. In parallel fashion, and according to plan, the same C2′ hydroxyl in compound **21** could be converted as its triethylsilyl ether (see compound **23**). *It will thus be recognized that these simply derived lactal derivatives 22 and 23 already carried the three "unique" (triethylsilyl) functions at the sites anticipated for 3-fold fucosylation.*

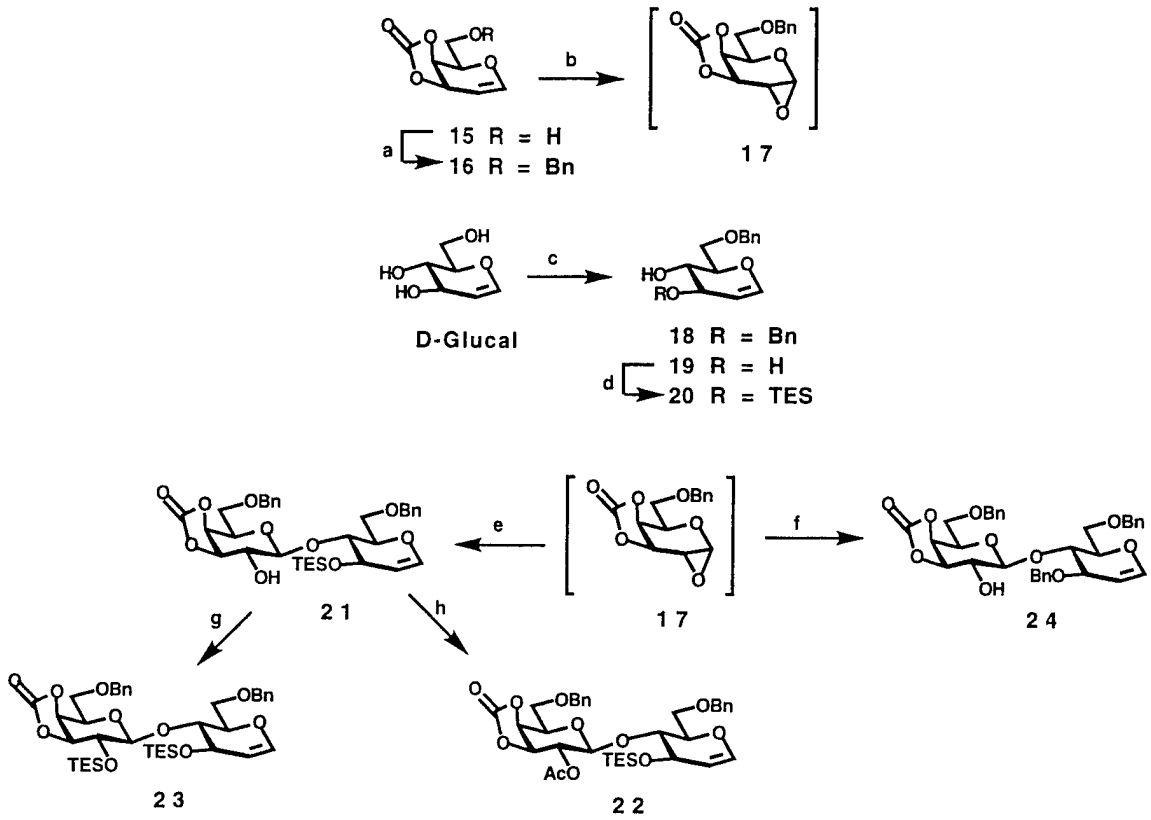
Similarly, couplings of dibenzyl glucal acceptor **18** with galactosyl donor **17** afforded a 55% yield of **24**. With disaccharide building blocks **22**, **23**, and **24** in hand, the program for reaching a hexasaccharide system, generalized as **4**, was carried forward.

We first turned to the merger of systems derived from **22** and **24**. In the latter case, it would be necessary to identify a specific glycosylation acceptor site at C3′ of a substituted galactal related to **24**. It will be recalled (see generalized structure **12** in Figure 3) that we had deliberately not specified the status of the neighboring oxygens at C2′ and C4′ in the galactose segment of the lactal (for **12**, R = protecting group or R = H). At first glance, it would seem to be necessary to develop specific protection at C2′ and C4′ of this acceptor moiety in order to pinpoint C3′ as the azaglycosyl acceptor site, and we were certainly prepared to pursue possibilities along these lines. A more exciting alternative view of the problem presented itself. *Perhaps even if C2′, C3′, and C4′ were all unprotected hydroxyl groups, the effective glycosyl acceptor site would be at C3′.* Certainly, there was encouraging precedent in the field of sialic acid acceptor sites[19] to suggest that a nondifferentiated vicinal triol of this type might be reasonably selective or even specific for reaction at C3′. To investigate this scenario for conciseness, compound **24** was subjected to basic conditions which served to cleave the cyclic carbonate, generating the potential triol acceptor **25** (see Scheme 2). Addition of *N*-iodobenzenesulfonamide to compound **22**, under the usual conditions, gave rise to the iodosulfonamide derivative **26**. Rearrangement of the sulfonamido function with concurrent thiolation was accomplished through the action of lithium hexamethyldisilazane in the presence of ethanethiol on compound **26**. This treatment was followed by acetylation (to restore any C3′ alcohol to the acetate state), thereby affording compound **27**. Thus, we had in hand the two components necessary to produce a tetrasaccharide.

In the event, glycosylation did occur at primarily C3′. Union of **25** and **27**, under the agency of methyl triflate in the presence of di-*tert*-butylpyridine, afforded compound **28** as the major product (see Scheme 3). Although this route constituted an extremely concise synthesis of tetrasaccharide **28**, the yield of this coupling event was problematic. The maximum yield obtained was 55%. More typical yields were in the range of 35−45%. Closer examination of the highly polar region of the chromatogram revealed an isomeric tetrasaccharide from a structure we tentatively assign as **29** (isolated in a ratio of 3:1−

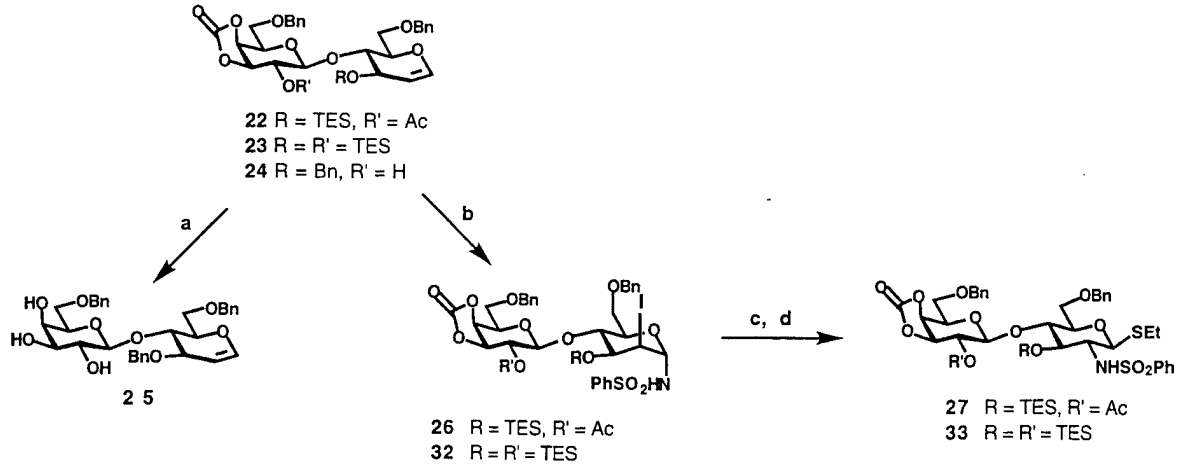
(19) (a) Kameyama, A.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1991**, *10*, 549. (b) Kondo, T.; Tomoo, T.; Abe, H.; Isobe, M.; Goto, T. *Chem. Lett.* **1996**, *5*, 337.

Scheme 1[a]



[a] Reagents: (a) NaH, DMF, BnBr (85%); (b) 3,3-dimethyldioxirane, CH_2Cl_2; (c) LHMDS, BnBr, DMF (30−40%); (d) TESCl, imidazole, CH_2Cl_2 (76%); (e) **20**, $ZnCl_2$, THF (65%); (f) **18**, $ZnCl_2$, THF (55%); (g) TESOTf, Et_3N, CH_2Cl_2 (92%); (h) Ac_2O, Et_3N, DMAP, CH_2Cl_2 (85%).

Scheme 2[a]



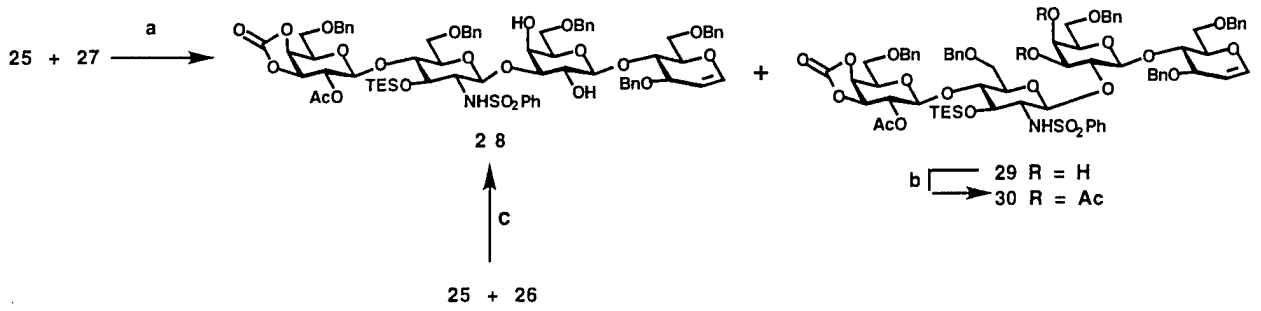
[a] Reagents: (a) K_2CO_3, MeOH (80%); (b) $I(coll)_2ClO_4$, $PhSO_2NH_2$, 4 Å MS, CH_2Cl_2 (81% for **26**, 92% for **32**); (c) LHMDS, EtSH, DMF (91% for **33**); (d) (only for **27**) Ac_2O, py, CH_2Cl_2 (95% over 2 steps).

2:1 favoring **28**). The massive polarity differences (R_f = 0.3 for **28** and 0.03 for **29** in 1:1 EtOAc−hexanes) between **28** and **29** presumably reflects different accessibilities of the two alcohol linkages to the silica surface in the two compounds. Acetylation of presumed **29** provided a triacetate assigned as **30.** Thus, the presumption that the C3′ hydroxyl in **27** would function as a fully regiospecific acceptor site turned out to be optimistic. However, synthetically useful selectivity in this reaction was achieved. While this route was certainly very concise in that it avoided the need for specific blocking functions at carbons 2′ and 4′ of the lactal derived from **24**, the formation of significant amounts of isomeric tetrasaccharide was cause for concern.

We hoped to improve upon the regiochemistry of this reaction by employing another glycosylation protocol that we had first demonstrated in a context of the synthesis of sialyl Le^x derivatives.[20] To implement this idea, we returned to the iodosulfonamide **26** with the hope that it itself might function as the eventual lactosamine donor. The glycosyl acceptor molecule would once again be compound **25**. However, here, the reaction would be mediated by bis(tri-*n*-butyltin)oxide in the presence of silver tetrafluoroborate. This reaction did, indeed, lead to an 84% yield of the previously encountered diol

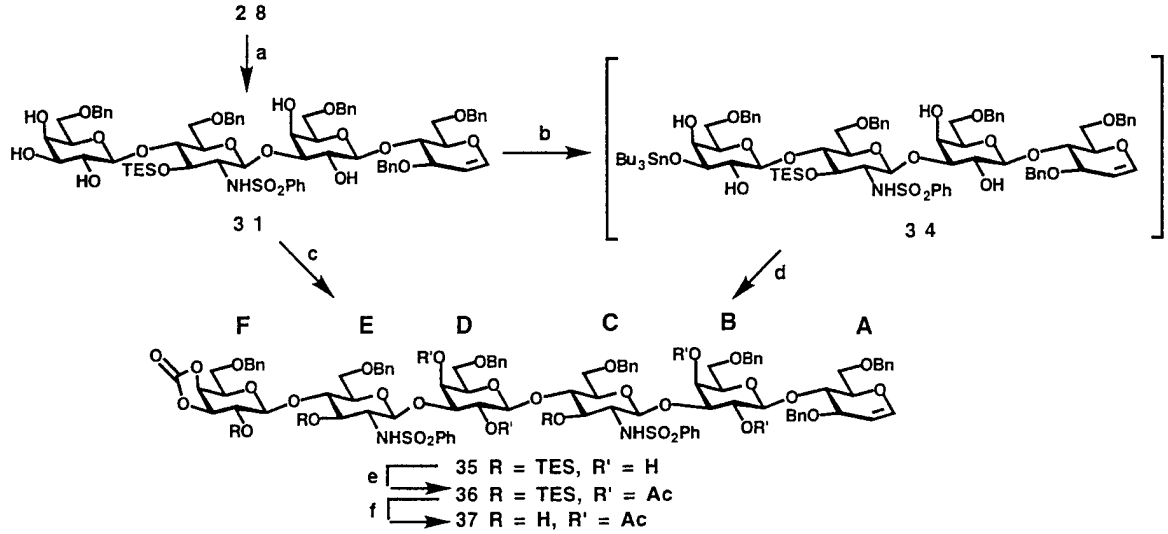
(20) Danishefsky, S. J.; Koseki, K.; Griffith, D. A.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Oriyama, T. *J. Am. Chem. Soc.* **1992**, *114*, 8331.

Scheme 3[a]



[a] Reagents: (a) MeOTf, di-*tert*-butylpyridine, Et_2O/CH_2Cl_2 (2:1), 4 Å MS (3:1−2:1 **28/29**, 35−55% for **28**); (b) Ac_2O, Py, DMAP, CH_2Cl_2 (95%); (c) (i) $(Bu_3Sn)_2O$ (2.2 equiv), PhH, **25** (4 equiv), reflux (ii) $AgBF_4$, THF, 4 Å MS (84%).

Scheme 4[a]



[a] Reagents: (a) K_2CO_3, MeOH (85%); (b) $(Bu_3Sn)_2O$, benzene, reflux; (c) **33**, MeOTf, di-*tert*-butylpyridine, Et_2O/CH_2Cl_2 (2:1), 4 Å MS (60%); (d) **32**, $AgBF_4$, THF, 4 Å MS (62%); (e) Ac_2O, Pyr, DMAP, CH_2Cl_2 (>95%); (f) TBAF/AcOH (93%).

tetrasaccharide **28**. While this direct "rollover" constituted a very pleasing and attractive solution to the assembly of **28**, this method is also not without its shortcomings in that it required recourse to excesses (4 equiv) of glycosyl acceptor **25** for high-yield coupling. In the absence of excess acceptor, it was difficult to drive the process to completion. On the other hand, the recovery of unused glycosyl acceptor, after the tin-mediated methodology, was very high (ca. 60−90%).

In summary on this point, the formation of tetrasaccharide **28** can be conducted through two methods. The reaction of donor **27** with acceptor **25**, in near stoichiometric equivalency, results in regioselective, but nonspecific, glycosylation at the desired C3′ site, giving rise to ca. 35−55% yields of tetrasaccharide. By contrast, recourse to "direct rollover" methodology, employing the tributylstannylated derivative of acceptor **25**, incurs the disadvantage of requiring significant excesses of acceptor. Since the recovery of the unreacted acceptor was high, we are currently favoring the direct method.

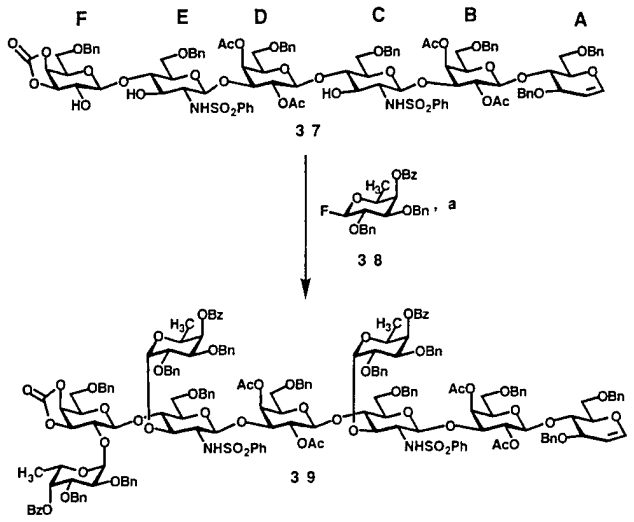
The next plateau to be reached was seen to be a hexasaccharide corresponding to a type **4** compound (see Figure 2). To further maximize the opportunities for conciseness, we were prepared to assume another significant risk in the use of undifferentiated hydroxyl groups in the acceptor site. The thought was to convert cyclic carbonate acetate **28** to pentaol **31**. This transformation was readily accomplished by treatment of **28** with potassium carbonate in methanol as shown in Scheme 4.

It was hoped that the glycosyl acceptor site of this *pentaol* would occur at the C3 hydroxyl group of the terminal galactose residue. The rationale was that the cluster of three neighboring and unprotected hydroxyl groups in the D ring in **31** would provide a greater locus of activity than the C2′ and C4′ hydroxyls of the B ring, each of which is flanked by two extensively substituted oxygens. Thus, the C2′ hydroxyl of the B ring is surrounded by the glycosidic bond to the A ring glucal as well as the glycosidic bond to the C ring glucosamine analogue. Similarly, the C4′ hydroxyl of the B ring abuts a benzyl ether protecting group at C6′ and is vicinal to a disaccharide group projecting from the oxygen from C3′.

Once again, we elected to explore the use of a thioethyl donor type to be derived from compound **23**. Toward this end, iodosulfonamidation of **23** afforded **32** which, under thiolate-mediated rearrangement of the sulfonamido group, gave rise to **33** (see Scheme 2). The coupling reaction between **28** and **33** was performed by the mediation of methyl triflate in the presence of di-*tert*-butylpyridine. Fortunately, in this case, the coupling could well be executed in acceptable yield (40−60%) without detectable formation of side product arising from glycosylation at alternative acceptor sites. In that sense, the situation was far better than in the 2 + 2 coupling of **25** + **27**, conducted through the same experimental protocol. However, we still experienced difficulties in obtaining reproducible yields.

For this reason, we elected again to examine the direct rollover method, this time using the tributylstannylated acceptor

Scheme 5[a]



[a] Reagents: (a) $Sn(OTf)_2$, di-*tert*-butylpyridine, Tol/THF (10:1), 4 Å MS (60%).

34 derived from pentaol **31** reacting with donor **32**. Using a 4-fold excess of **31** (once again unused acceptor was recovered in very high yield), a 62% yield of **35** was obtained. As before, the direct method lent itself to much more reproducible chemistry which compensated for the need to recover unused acceptor. This is the method we currently follow.

We were now ready to address the culminating stage of the plan, namely, the construction of the nonasaccharide. The preparation for this purpose simply involved cleavage of the unique (silyl) protecting groups (see system **3**, Figure 2). We had carefully prepared for the possibility of such a step by carrying relatively dischargable triethylsilyl protecting groups at the oxygens destined for fucosylation. Indeed, treatment of compound **36** with buffered tetrabutylammonium fluoride provided triol **37**. As our L-fucosyl donor, we employed compound **38**[21] which had been used several times before[5] in our program to synthesize blood group determinants and tumor antigens.

The critical step of the synthesis, *3-fold fucosylation*, was indeed accomplished through the reaction of **37** and **38**. A 60% yield of nonasaccharide **39** was obtained (see Scheme 5). Needless to say, we did not have available to us any substance for direct comparison, or any downstream product which might be used in a strategy to confirm the structure of **39**. The structure assignment would not be fully clarified until the synthesis was concluded and even then without benefit of a reference sample. Therefore, it was important to marshal convincing evidence that we had indeed generated the compound we were claiming, i.e., structure **39**. Our strategy in this regard was to return to compound **37**. If its structure could be established, we would be confident in concluding that the three fucosyl monosaccharides had been introduced at the proper positions. NMR analysis at the eight anomeric centers in the nonasaccharide, in conjunction with the information we had already accumulated at the hexa stage, would provide acceptable support of the structure of the trifucosylated product **39**.

The examination of the NMR and mass spectra of compound **37** indicated the presence of the expected four acetate groups. Fortunately NMR experiments could be used to assign the positions of these acetates. The sites of the free hydroxyl groups would thus have been established by difference.

The locations of the four acetate protecting groups were determined unequivocally through the use of several NMR experiments. The four multiplets between 5.0 and 5.45 ppm fall in the distinctive region for the protons on *O*-acetylated sugar carbons. The multiplets at 5.06 and 5.16 ppm were assigned to the H-2 protons of either sugar ring B or ring D, on the basis of their coupling patterns, and their cross peaks in the double quantum COSY experiment (DQCOSY) to protons assumed to be at anomeric carbons on the basis of chemical shifts.

The confirmation that these signals indeed arise from anomeric protons followed from the $^1H-^{13}C$ correlation experiments, where these resonances show cross peaks to carbons with the distinct anomeric carbon shift.[22] These H-2 protons, which had now been identified, show DQCOSY cross peaks to one other vicinal partner each, which must be the respective H-3's for the two pyranose rings. The peaks at 5.36 and 5.40 ppm arise from the two other sites of *O*-acetylation, i.e., at the axial hydroxyls at C4 of the rings B and D. That these centers carry the remaining acetoxy groups can be surmised from the correlation of these protons to carbonyl resonances in long-range (HMBC) $^1H-^{13}C$ correlation experiments.

Analysis of the DQCOSY experiment showed that three additional sites have cross peaks to respective H-3 protons identified from connection to the H-2 resonances. These data in the aggregate prove that the acetylation sites on sugar rings B and D are on carbons 2 and 4. The data confirm that the glycosylation linkages formed during the reaction between components **25** and **27** (or **25** and **26**) as well as between **31** and **33** (or **32** and **34**) have occurred through acceptor hydroxyl sites at C-3 of the respective residues. Using the markers for rings B and D that come from the acetates, along with the characteristic ^1H and ^{13}C shifts of the glycal, and data from the long-range $^1H-^{13}C$ correlation (HMBC) experiment, it was possible to trace the through bond connectivities around the respective sugar rings and across the glycosidic linkages to confirm the desired assembly sequence of the compound **37**. Also, all of the glycosidic linkages in compound **37** were confirmed as β-linkages on the basis of the splitting in the proton dimension of the individually resolved anomeric cross peaks in the HMQC experiment. These exhibited a coupling constant for the anomeric H-1 protons of 6.00 Hz or higher. It is on these bases that we were confident of the assignments for compounds **37** and **39**. The latter would now be advanced to our goal structures.

At this point, our remaining objectives were (i) the total synthesis of the KH-1 antigen itself, (ii) the synthesis of a form of the epitope which would be suitable for conjugation to carrier program, and (iii) the synthesis of a truncated construct which would help in ascertaining antibody specificities. In terms of reaching goals (i) and (ii) we were now in a position starting with the nonasaccharide glycal **39** to take advantage of previous experiences in the syntheses of glycolipids, particularly those in the Globo-H (breast tumor antigen) construct.[6]

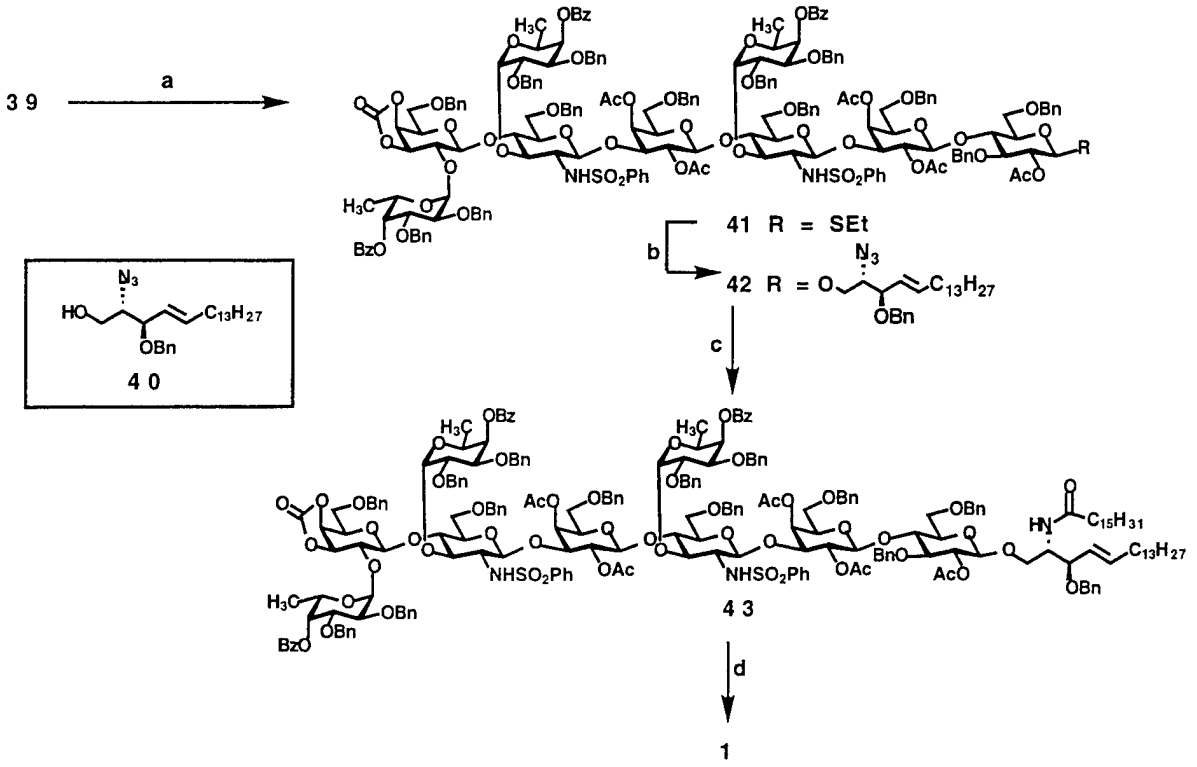
Our first step in the proposed introduction of the ceramide side chain to reach the natural KH-1 antigen (**1**) was the epoxidation of glycal **39**. While this reaction seemed to occur smoothly, attempts to use the epoxide directly as a glycosyl donor with respect to the well-known preceramide acceptor **40**[23] led to low yields of coupling. These difficulties perhaps arise from dominant conformers of a nonasaccharide epoxide in which required access to the anomeric oxido carbon by glycosyl

(21) Danishefsky, S. J.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Koseki, K.; Oriyama, T.; Griffith, D. A.; Wong, C.-H.; Dumas, D. P. *J. Am. Chem. Soc.* **1992**, *114*, 8329.

(22) Lerner, L.; Bax, A. *Carbohydr. Res.* **1987**, *166*, 35.

(23) Schmidt, R. R.; Zimmermann, P. *Tetrahedron Lett.* **1986**, *27*, 481.

Scheme 6[a]



[a] Reagents: (a) (i) 3,3-dimethyldioxirane, CH_2Cl_2; (ii) EtSH, CH_2Cl_2, H^+(cat); (iii) Ac_2O, Py, CH_2Cl_2 (60% 3 steps); (b) **40**, MeOTf, Et_2O/CH_2Cl_2 (2:1), 4 Å MS (55%); (c) Lindlar's catalyst, H_2, palmitic anhydride, EtOAc (85%); (d) (i) Na^0, NH_3, THF; MeOH quench; (ii) Ac_2O, Et_2N, DMAP, DMF, THF; (iii) MeONa, MeOH (70% 3 steps).

acceptor **40** was difficult. Given the chemical instability of glycal epoxides to Lewis acids, relatively slow glycosylation would result in low yields of glycoside, through loss of competence of the donor moiety.

Accordingly, we turned to the application of a recently developed variation of the glycal epoxy donor method.[24] This protocol started with epoxidation, followed by thiolation of the resulting epoxide of **39** and further acetylation in the usual way, leading to acetate **41** (see Scheme 6). With expectation of effective neighboring group participation of the C2 acetoxyl function available to guide a β-glycosidation, compound **41** was treated with **40** under the agency of methyl triflate. This process indeed led to the formation of glycoside **42**, in 55% yield.

From this point, the required methodology was quite familiar to us.[6] Reduction of the azide **42** was coordinated with palmitoylation of the resultant amine generated, in situ (see compound **43**). Cleavage of all aromatic groups was apparently accomplished by treatment of **43** with sodium in liquid ammonia. The resultant product was quenched with methanol. Under the basic conditions thus employed, all of the esters (including the cyclic carbonate) were cleaved. To regularize the blocking groups (vide infra), the resultant material was peracetylated. Finally, cleavage of all acetates was accomplished with sodium methoxide in methanol to give rise to the pure KH-1 antigen (**1**).

The structure assignment of the fully synthetic material is supported by a mass spectral measurement which confirmed the molecular formula. More telling was the very close correspondence of the NMR data measured on fully synthetic **1**, with the fragmentary data previously reported for the naturally occurring material. Most compelling was the extensive and self-consistent NMR analysis which had been conducted on intermediates en route to, and including, **39** (particularly compound **37**). Also, a fully rigorous NMR-based verification was possible at the stage of allyl glycoside **44**, whose synthesis was accomplished from the same **39** (vide infra).

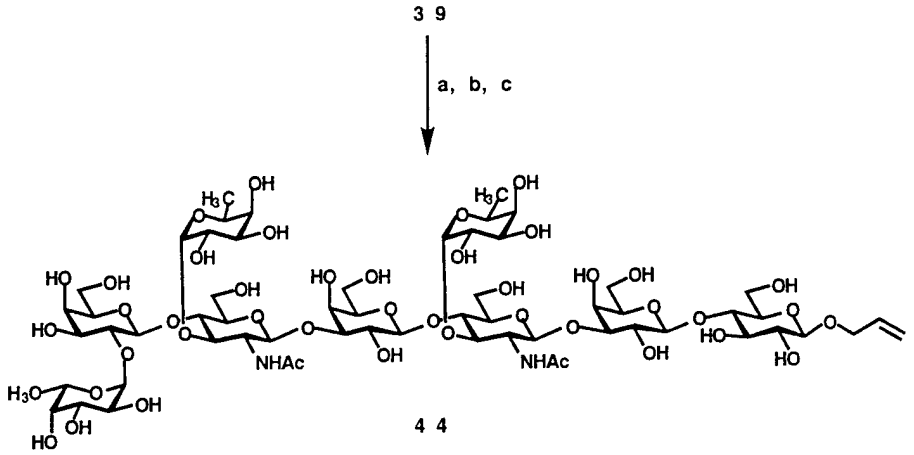
For our bioconjugation studies, we identified allyl glycoside **44** as our goal system. For this purpose we returned to the glycal **39**. Again, discharge of all the aromatic groups was accomplished by treatment of compound **39** with sodium in liquid ammonia (see Scheme 7). The remaining esters and the cyclic carbonate were cleaved after the reaction was quenched with methanol. The crude product was subjected to exhaustive acetylation, followed by epoxidation in the usual way with dimethyldioxirane. The resultant epoxide was opened by solvolysis with allyl alcohol. The crude product was fully deacylated through the action of sodium methoxide in methanol to afford compound **44** in 60% overall yield.

Conjugation of **44** to carrier keyhole lympet hemocyanin (KLH) protein was accomplished through ozonolysis of the double bond of the allyl group. This treatment was followed by reductive amination of the resultant aldehyde to KLH. The procedure for accomplishing conjugation to KLH is detailed in the Experimental Section.

In preparing for immunological investigations, it would be helpful to determine the specificities of various antibodies to the structural features of the KH-1 antigen. Toward that end, it was of interest to generate truncated structures in which segments of the molecule would be deleted. In our first such effort, we directed our attention to a construct in which the three fucose residues as well as the *N*-acetyl function on the E ring would be retained (see compound **50**). However, the terminal *N*-acetylactosamine substructure would be deleted. For this purpose, a modified synthesis was initiated. We returned to

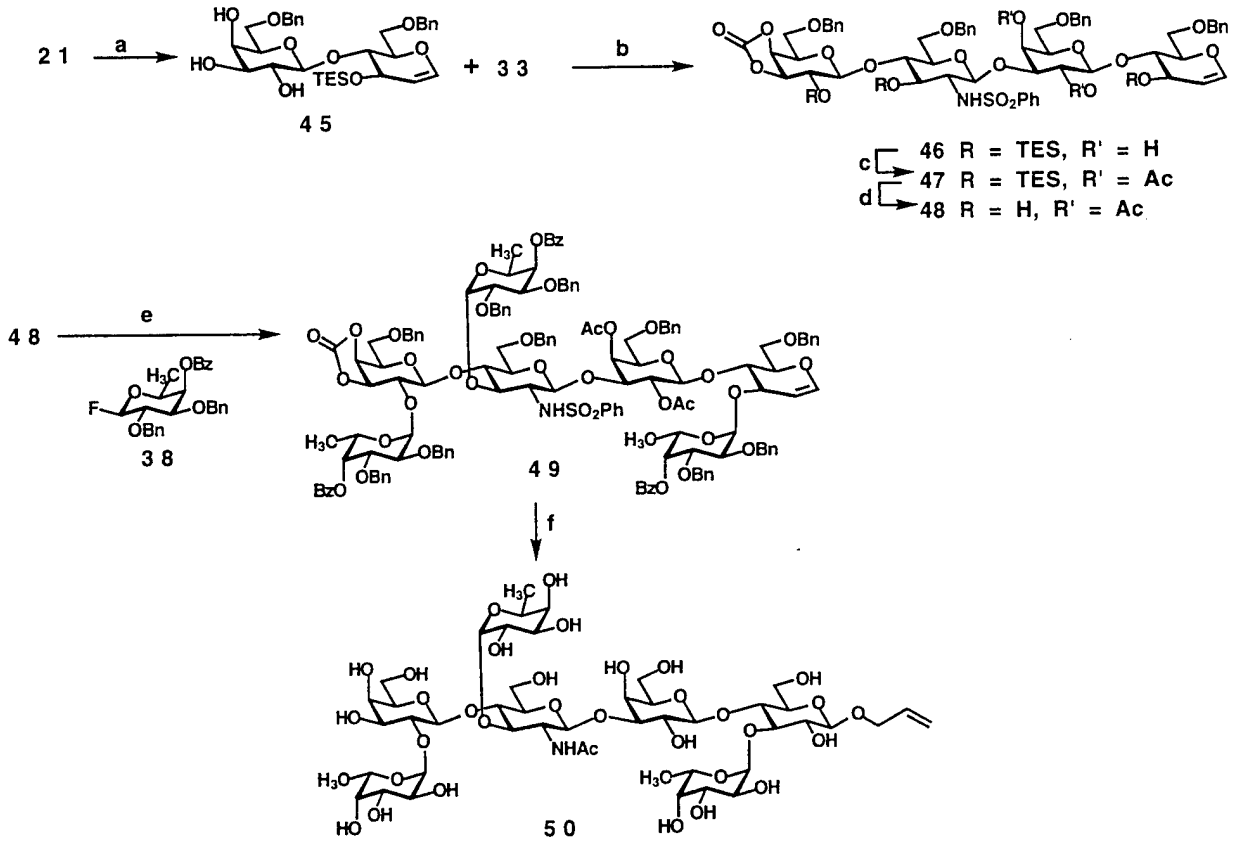
(24) Seeberger, P. H.; Eckhardt, M.; Gutteridge, C. E.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 10064.

Scheme 7[a]



[a] Reagents: (a) (i) Na^0, NH_3, THF; (MeOH quench); (ii) Ac_2O, Et_3N, DMAP, DMF, THF; (b) (i) 3,3-dimethyldioxirane, CH_2Cl_2; (ii) allyl alcohol; (c) MeONa, MeOH (60% 3 steps).

Scheme 8[a]



[a] Reagents: (a) K_2CO_3, MeOH (quant); (b) MeOTf, di-*tert*-butylpyridine, Et_2O/CH_2Cl_2 (2:1), 4 Å MS (61%); (c) Ac_2O, Py, DMAP, CH_2Cl_2 (93%); (d) 1:1 TBAF/AcOH (72%); (e) $Sn(OTf)_2$, Tol/THF (10:1), 4 Å MS (39%); (f) (i) Na^0, NH_3, THF; MeOH quench; (ii) Ac_2O, Et_3N, DMAP, THF, DMF; (iii) 3,3-dimethyldioxirane, CH_2Cl_2; (iv) allyl alcohol; (v) MeONa, MeOH (60% 3 steps).

compound **21** which would first serve as an acceptor substructure. Cleavage of the cyclic carbonate linkage gave rise to compound **45** (see Scheme 8).

The donor, to be coupled to compound **45**, was the C1 thioethyl C2 phenylsulfonamide system **33**, containing the two unique TES protecting groups in the E and F rings. Coupling of **45** with **33** occurred under the usual conditions to give an acceptable 60% yield of the tetrasaccharide **46**. Acetylation of **46** afforded a diacetate, **47**. This system now contained the three uniquely identified triethylsilyl ether functions which were cleaved through the action of TBAF (see compound **48**). Once again, it was possible to conduct 3-fold fucosylation. This chemistry was not optimized, and we accepted a 39% yield of compound **49**. The remaining steps for conversion of **49** to allyl glycoside **50** ran parallel to those used with related structures in the synthesis of compound **44**.

With serviceable routes to the various tetrasaccharide, disaccharide, and monosaccharide moieties contained in the KH-1 construct, it seems likely that other permuted structures can be readily assembled. These can be used to ascertain epitope specificities for monoclonal antibodies which are being elicited as part of this vaccine-oriented program.

Summary

All of the chemical goals identified at the outset of this project have been realized. The KH-1 antigen (**1**) has been synthesized. It has also been synthesized in conjugatable form for delivery to carrier protein (see compound **44**). Conjugation to KLH has, in fact, been achieved (see the Experimental Section). A format for synthesizing truncated versions of the KH-1 antigen in order to establish epitope specificities for various monoclonal antibodies being harvested has also been achieved (see compound **50**).

From a strategic standpoint, the strategy of 3-fold fucosylation to achieve conciseness in the synthesis was certainly vindicated. Also, the synthesis speaks forcefully to the advantages of glycal assembly and to the minimization of protecting group manipulations for purposes of differentiating functional groups. Thus, in three instances (see compounds **25**, **31**, and **45**) we took recourse to glycosyl acceptors with more than one potential glycosylation site. In the latter two cases, coupling was accomplished under nearly stoichiometric conditions utilizing 1-β-thioethyl-2α-phenylsulfonamido donors under the agency of methyl triflate. In the case of coupling of acceptors **25** and **31**, these delicate reaction conditions led to some difficulties in reproducing the best case yield. However, excellent yields in a highly reproducible fashion were obtained by treatment of the 1α-sulfonamido-2β-iodo donor type (see compounds **26** and **32**), utilizing a stannylated version of acceptor (**25** and **31**). These reactions, however, require a significant excess of glycosyl acceptor. Since the recovery yields are very high, our current technology favors this practice.

The primary focus of the KH-1 antigen problem has now shifted to issues of immunology and vaccinology. Data on these matters will soon be forthcoming.

Experimental Section

General Methods. All commercial materials were used without further purifications unless otherwise noted. The following solvents were distilled under positive pressure of dry nitrogen immediately before use: THF and ether from sodium−benzophenone ketyl, and CH_2Cl_2, toluene, and benzene from CaH_2. All the reactions were performed under N_2 atmosphere. NMR (^1H, ^{13}C) spectra were recorded on a Bruker AMX-400 MHz spectrometer, a Bruker Avance DRX-500 MHz spectrometer, and a Varian 600 Unity plus 600 MHz spectrometer, referenced to TMS (^1H NMR, δ 0.00) or $CDCl_3$ (^{13}C NMR, δ 77.0) and CD_3OD(^{13}C NMR, δ 49.05) peaks unless otherwise stated. LB = 1.0 Hz was used before Fourier transformation for all of the ^{13}C NMR. IR spectra were recorded with a Perkin-Elmer 1600 series FTIR spectrometer, and optical rotations were measured with a JASCO DIP-370 digital polarimeter using a 10 cm path length cell. Low- and high-resolution mass spectral analyses were performed with a JEOL JMS-DX-303 HF mass spectrometer. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate−ammonium molybdate solution followed by heating. Flash column chromatography was performed using the indicated solvent on E. Merck silica gel 60 (40−63 μm) or Sigma H-Type silica gel (10−40 μm) for normal phase and EM Science Lichroprep RP-18 (15−25 μm) for reverse phase. Melting points are obtained with an Electrothermal melting point apparatus (series no. 9100) and are uncorrected.

6-*O*-Benzyl 3,4-*O*-Carbonate 16. To a solution of the galactal carbonate derivative **15** (5.36 g, 34.4 mmol) in dry DMF (50 mL) at 0 °C was added benzyl bromide (12.3 mL, 103 mmol), followed by NaH (60% oil dispersion, 1.50 g, 1.1 equiv). The reaction mixture was stirred for 1 h, diluted with $CHCl_3$ (50 mL), treated with brine solution (20 mL), and again stirred for 5 min. The organic layer was separated, dried ($MgSO_4$), and filtered. The filtrate was concentrated to afford a syrup. Chromatography with 1:1 EtOAc/hexanes afforded 7.66 g of compound **16** (85%) as a syrup: $[\alpha]^{23}_D = -92.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3030, 2875, 1797, 1647, 1496, 1453, 1371, 1244, 1164, 1110, 1010, 837, 699 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.7−3.9 (m, 2H, H-6), 4.08 (br t, 1H, J = 7.4 Hz, H-5), 4.58 (s, 2H, $-CH_2Ar$), 4.90 (d, 1H, J = 7.8 Hz, H-4), 4.93 (br m, 1H, H-3), 5.14 (dd, 1H, J = 3.2, 7.7 Hz, H-2), 6.66 (d, 1H, J = 6.2 Hz, H-1), 7.28−7.45 (m, 5H, Ar−H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 67.97, 68.74, 72.41, 73.14, 73.66, 97.97, 127.77, 127.93, 128.44, 137.18, 149.06, 153.98; HRMS (FAB) calcd for $C_{14}H_{14}O_5$·Na $(M + Na)^+$ 285.084, found 285.072.

6-*O*-Benzylglucal (19). To a solution of D-glucal (10.0 g, 68.4 mmol) in dry DMF (200 mL) was added LHMDS (1.0 M solution in THF, 75.3 mL, 1.1 equiv) dropwise at −40 °C, followed by BnBr (8.18 mL, 68.4 mmol). The solution was stirred mechanically for 6 h, allowing the temperature to rise to 0 °C. Saturated solution of NH_4Cl (50 mL) was added to the reaction mixture, followed by EtOAc (200 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 × 50 mL). Combined organic layers were washed with brine (50 mL) and water (50 mL), dried with $MgSO_4$, filtered, concentrated, and submitted for column chromatography (1:1 EtOAc/hexanes) to obtain compound **19** as a syrup (4.80 g, 30%): $[\alpha]^{23}_D = +11.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3342, 2871, 1642, 1656, 1231, 1101, 1027, 851, 738 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.6−3.85 (m, 5H,), 4.06 (d, 1H, J = 4.0 Hz, −OH), 4.11 (br t, 1H, H-3), 4.46 (d, 1H, J = 12.0 Hz, $-CH_2Ar$), 4.52 (d, 1H, J = 12.0 Hz, $-CH_2$-Ar), 4.57 (dd, 1H, J = 1.8, 6.0 Hz, H-2), 6.21 (d, 1H, J = 6.0 Hz, H-1), 7.15−7.35 (m, 5H, Ar−H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 69.06, 69.63, 70.28, 73.427, 76.95, 102.72, 127.29, 127.55, 128.21, 128.24, 137.60, 137.75, 143.86.

6-*O*-Benzyl-3-*O*-(triethylsilyl)glucal (20). To a solution of compound **19** (4.00 g, 16.8 mmol) in dry CH_2Cl_2 (70 mL) were added imidazole (1.26 g, 18.5 mmol) and DMAP (0.01 g), and the solution was cooled to −78 °C. At −78 °C, to the reaction mixture was added TESCl (3.10 mL, 18.5 mmol) dropwise, and the resulting solution was allowed to warm to −40 °C. The reaction mixture was stirred for 4 h, diluted with EtOAc (200 mL), and washed with water (2 × 100 mL), a saturated solution of $NaHCO_3$ (3 × 100 mL), and brine (10 mL). The organic layer was dried ($NaSO_4$), filtered, concentrated, and submitted for column chromatography (1:9 EtOAc/hexanes) to provide **20** (4.46 g,76%) as a syrup: $[\alpha]^{22}_D = +44.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3468, 3030, 2953, 2875, 1644, 1453, 1237, 1086, 871, 737 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.55 (q, 6H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.88 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 2.47 (d, 1H, J = 4.1 Hz, −OH), 3.6−3.75 (m, 3H, 2H-6, H-4), 4.13 (br d, 1H, J = 6.4 Hz, H-3), 4.47 and 4.52 (2d, 2H, J = 12.0 Hz, $-CH_2Ar$), 4.55 (dd, 1H, J = 2.2, 6.2 Hz, H-2), 6.21 (d, 1H, J = 6.0 Hz, H-1), 7.10−7.40 (m, 5H, Ar−H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 4.84, 6.66, 69.05, 69.64, 70.56, 73.47, 76.97, 103.44, 127.59, 127.64, 128.27, 137.78, 143.33; HRMS (FAB) calcd for $C_{19}H_{30}O_4Si$·Na $(M + Na)^+$ 373.180, found 373.181.

6,6′-Di-*O*-benzyl 3′,4′-Carbonate 3-*O*-Triethylsilyl Lactal Derivative 21. To a solution of compound **16** (2.99 g, 11.4 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C was added 3,3-dimethyldioxirane (300 mL, 0.0800 M solution in acetone). The reaction mixture was stirred at 0 °C for 1 h. The solvent was evaporated by N_2, and further dried in vacuo for 10 min. The resulting 1,2-anhydro sugar **17** was dissolved in a solution of compound **20** (6.00 g, 17.1 mmol) in dry THF (30 mL), and at 0 °C to the resulting solution was added a 1.0 M solution of $ZnCl_2$ in ether (5.70 mL, 0.5 equiv). The reaction mixture was stirred at room temperature for 24 h, diluted with EtOAc (50 mL), and washed with a saturated solution of $NaHCO_3$ (2 × 10 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (2:3 EtOAc/hexanes) to provide compound **21** (4.76 g, 66%) as a syrup: $[\alpha]^{22}_D = -25.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3439, 3030, 2910, 1804, 1725, 1647, 1453, 1371, 1243, 1074, 847, 741 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.58 (q, 6H, J = 8.0 Hz, $-SiCH_2CH_3$), 0.92 (t, 9H, J = 8.0 Hz, $-SiCH_2CH_3$), 3.51 (d, 1H, J = 2.8 Hz, −OH), 3.62 (ddd, 1H, J = 2.8, 7.2, 7.2 Hz, H-2′), 3.65−3.75 (m, 3H), 3.85 (m, 1H), 3.93 (dd, 1H, J = 4.9, 11.2 Hz), 3.99 (br t, 1H, J = 5.3, 6.48 Hz), 4.09 (br m, 1H), 4.27 (br t, 1H, J = 4.2 Hz), 4.48−4.68 (m, 6H, $-CH_2Ar$), 4.70 (dd, 1H, J = 3.4, 6.2 Hz, H-2), 4.74 (dd, 1H, J = 1.8, 7.2 Hz, H-4), 6.32 (d, 1H, J = 6.0 Hz, H-1), 7.2−7.4 (m, 10H, Ar−H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 4.75, 6.67, 65.65, 67.79, 67.93, 70.42, 71.49, 73.43, 73.58, 74.46, 75.27, 75.42, 78.05, 99.94, 102.61,

127.79, 127.85, 128.14, 128.33, 137.36, 137.53, 143.00, 153.96; HRMS calcd for $C_{33}H_{44}O_{10}Si$·Na (M + Na)$^+$ 651.170, found 651.368.

2′-*O*-Acetyl-6,6′-di-*O*-benzyl 3′,4′-Carbonate 3-*O*-Triethylsilyl Lactal (22). To a solution of compound **21** (3.50 g, 5.50 mmol) in CH_2Cl_2 (30 mL) were added Et_3N (3 mL), Ac_2O (3 mL), and a catalytic amount of DMAP. The reaction mixture was stirred overnight, diluted with EtOAc (50 mL), and washed with saturated solutions of $CuSO_4$ (3 × 10 mL), water (1 × 10 mL), $NaHCO_3$ (2 × 10 mL), and brine (1 × 10 mL) sequentially. The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted to chromatography (1:1 EtOAc/hexanes) to provide **22** in quantitative yield: $[\alpha]^{23}_D = -42.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 2954, 2875, 1809, 1755, 1646, 1454, 1222, 1060, 743 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.58 (q, 6H, *J* = 7.9 Hz, $-SiCH_2$-CH_3), 0.92 (t, 9H, *J* = 7.9 Hz, $-SiCH_2CH_3$), 2.06 (s, 3H, $-COCH_3$), 3.63 (dd, 1H, *J* = 2.9, 10.9 Hz, H-5), 3.70 (br d, 2H, *J* = 10.5 Hz, 2H-6), 3.85 (dd, 1H, *J* = 6.1 Hz, *J* = 10.92, H-5′), 3.9−4.0 (m, 2H, 2H-6′), 4.10−4.2 (m, 2H), 4.5−4.6 (m, 4H, 2-CH_2Ar), 4.64 (dd, 1H, *J* = 4.0, 8.0 Hz, H-3′), 4.71 (dd, 1H, *J* = 4.2, 5.8 Hz, H-2), 4.84 (dd, 1H, *J* = 1.0, 8.1 Hz, H-4′), 4.90 (d, 1H, *J* = 4.6 Hz, H-1′), 4.99 (t, 1H, *J* = 4.2 Hz, H-4′), 6.30 (d, 1H, *J* = 6.2 Hz, H-1), 7.15−7.40 (m, 10H, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.78, 6.73, 20.58, 64.78, 67.94, 67.99, 69.38, 69.50, 73.19, 73.35, 73.79, 73.92, 74.56, 74.86, 96.82, 102.27, 127.60, 127.75, 127.78, 127.93, 128.29, 128.44, 137.35, 138.01, 142.99, 153.27, 168.54; HRMS calcd for $C_{35}H_{46}O_{11}Si$·Na (M + Na)$^+$ 693.270, found 693.273.

6,6′-Di-*O*-benzyl 3′,4′-Carbonate 2′,3-Bis(*O*-triethylsilyl) Lactal (23). To a solution of the lactal **21** (3.00 g, 4.77 mmol) in dry CH_2Cl_2 (50 mL) was added Et_3N (3.34 mL) followed by dropwise addition of TESOTf (1.61 mL, 7.15 mmol) at 0 °C. The reaction mixture was stirred for 3 h, and washed with a saturated solution of $NaHCO_3$ (2 × 15 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (1:4 EtOAc/hexanes) to provide **23** (3.27 g, 92%) as a syrup: $[\alpha]^{23}_D = -38.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3087, 2953, 2875, 1819, 1647, 1647, 1454, 1365, 1240, 1101, 854, 739 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.57 and 0.617 (2q, 12H, *J* = 8.0 Hz, $-SiCH_2CH_3$), 0.92 and 0.94 (2t, 18H, *J* = 8.0 Hz, $-SiCH_2CH_3$), 3.5−3.75 (m, 4H), 3.8−4.0 (m, 3H), 4.05−4.20 (m, 2H), 4.49 (dd, 1H, *J* = 4.4, 7.2 Hz), 4.50−4.62 (m, 4H, $-CH_2$Ar), 4.64 (d, 1H, *J* = 5.2 Hz, H-1′), 4.70 (dd, 1H, *J* = 4.0, 5.6 Hz, H-4′), 4.76 (br d, 1H, *J* = 7.5 Hz, H-2), 6.32 (d, 1H, *J* = 6.0 Hz, H-1); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 4.56, 4.79, 6.58, 6.76, 65.24, 67.99, 68.02, 69.48, 71.06, 73.37, 73.76, 74.24, 74.37, 75.10, 78.21, 99.21, 99.34, 102.56, 127.63, 127.77, 127.79, 127.88, 128.32, 128.43, 137.53, 138.09, 143.08, 153.87; HRMS calcd for $C_{39}H_{58}O_{10}Si_2$·Na 765.360 (M + Na)$^+$, found 765.347.

3,6,6′-Tri-*O*-benzyl 3′,4′-Carbonate Lactal (24). To a solution of compound **16** (2.99 g, 11.4 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C was added 3,3-dimethyldioxirane (300 mL, 0.0800 M solution in acetone). The reaction mixture was stirred at 0 °C for 1 h. The organic solvent was evaporated by N_2 stream, and further dried in vacuo for 10 min. The resulting 1,2-anhydro sugar **17** was dissolved in a solution of known 3,6-di-*O*-benzylglucal (**18**) (5.29 g, 17.2 mmol) in dry THF (30 mL). To the solution at 0 °C was added a 1.0 M solution of $ZnCl_2$ in ether (5.71 mL, 0.5 equiv). The reaction mixture was stirred at room temperature for 24 h, diluted with EtOAc (50 mL), and washed with a saturated solution of $NaHCO_3$ (2 × 10 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (1:1 EtOAc/hexanes) to provide compound **24** (3.31 g, 48%) as a syrup: $[\alpha]^{22}_D = -38.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3437, 3029, 2871, 1804, 1648, 1453, 1367, 1166, 1097, 1027, 739, 697 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.55−3.62 (m, 2H), 3.62−3.70 (m, 2H), 3.70−3.78 (m, 2H), 3.95−4.11 (m, 2H), 3.95−4.11 (m, 2H), 4.17 (dd, 1H, *J* = 5.4, 7.0 Hz), 4.27 (ddd, 1H, *J* = 1.1, 1.7, 5.3 Hz), 4.44 (s, 2H, $-CH_2$Ar), 4.77 (dd, 1H, *J* = 2.5, 6.1 Hz, H-2), 6.28 (d, 1H, *J* = 6.0 Hz, H-1), 7.10−7.40 (m, 15H, Ar−H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 68.00, 68.09, 70.55, 70.63, 72.20, 73.58, 73.81, 74.58, 74.82, 75.26, 76.18, 78.47, 100.17, 101.32, 127.43 (2C), 127.56, 127.72 (2C), 127.83, 127.90, 128.00 (2C), 128.31 (2C), 128.37 (2C), 128.44 (2C), 137.28, 137.43, 138.29, 144.59, 153.97; HRMS calcd for $C_{34}H_{36}O_{10}$·Na (M + Na)$^+$ 627.220, found 627.220.

3,6,6′-Tri-*O*-benzyllactal (25). To a solution of compound **24** (3.00 g, 4.96 mmol) in MeOH (100 mL) was added a solution of sodium methoxide (1.00 mL, 25% by weight in MeOH) dropwise. The reaction mixture was stirred for 1 h. The volatiles were removed in vacuo. The residual syrup obtained was quickly purified by column chromatography (2.5% MeOH in EtOAc) to provide 2.68 g (91%) of **25** as a syrup: $[\alpha]^{22}_D = -14.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3415, 3029, 2867, 1647, 1453, 1246, 1068, 735 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.48−3.56 (m, 2H), 3.62 (dd, 1H, *J* = 4.8, 8.0 Hz), 3.66−3.78 (m, 3H), 3.91 (d, 1H, *J* = 4.4 Hz), 3.97 (dd, 1H, *J* = 4.0, 8.8 Hz), 4.18−4.28 (m, 4H), 4.47 (s, 2H, $-CH_2$Ar), 4.52 (d, 1H, *J* = 8.0 Hz), 5.59 (s, 1H, $-CH_2$Ar), 4.57−4.65 (m, 2H, $-CH_2$Ar), 4.85 (dd, 1H, *J* = 2.4, 4.8 Hz, H-2), 6.41 (d, 1H, *J* = 4.8 Hz, H-1), 7.20−7.45 (m, 15H, Ar−H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 67.92, 68.86, 69.19, 69.82, 71.53, 73.35 (2C), 73.39, 73.42, 73.87, 76.26, 100.01, 103.30, 127.35, 127.42, 127.59, 127.74, 128.19, 128.29, 137.72, 137.81, 138.52, 144.57; HRMS (FAB) calcd for $C_{33}H_{38}O_9Na$ (M + Na)$^+$ 601.240, found 601.242.

Iodo Sulfonamide Disaccharide 26. To a solution of compound **22** (2.50 g, 3.72 mmol) with 4 Å molecular sieves (3.00 g) and benzenesulfonamide (2.92 g, 18.6 mmol) in CH_2Cl_2 at 0 °C was added a solution of I(*sym*-coll)$_2ClO_4$ [freshly prepared from Ag(coll)$_2ClO_4$ (8.36 g, 18.6 mmol) and I_2 (4.53 g, 18.5 mmol)] in CH_2Cl_2 (40 mL) via cannula. The ice bath was removed, and the reaction mixture was stirred at room temperature for 1 h. The resulting suspension was filtered through a pad of silica gel, and the filtrate was washed with a saturated solution of $Na_2S_2O_3$ (3 × 25 mL), followed by a saturated solution of $CuSO_4$ (5 × 25 mL) and H_2O (2 × 10 mL). The separated organic layer was dried ($MgSO_4$), filtered, concentrated, and submitted for column chromatography (5% EtOAc in CH_2Cl_2, start gradient) to obtain **26** (2.87 g, 81%) as a syrup: $[\alpha]^{23}_D = -30.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3267, 2954, 1806, 1755, 1495, 1458, 1370, 1342, 1090, 813, 750 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.66 (m, 6H, *J* = 7.8 Hz, $-SiCH_2$-CH_3) 0.95 (t, 9H, *J* = 7.8 Hz, $-SiCH_2CH_3$), 2.04 (s, 3H, $-COCH_3$), 3.44 (dd, 1H, *J* = 5.6, 10.2 Hz, H-5), 3.55−3.72 (m, 4H), 3.86 (br s, 1H), 4.11 (t, 1H, *J* = 7.0 Hz), 4.23 (br s, 1H), 4.35 (dd, 1H, *J* = 2.3, 10.0 Hz), 4.44 and 4.50 (2d, 2H, *J* = 11.9 Hz, $-CH_2$Ar), 4.57 (s, 2H, $-CH_2$Ar), 4.70 (br d, 1H, *J* = 8.3 Hz), 4.89 (br s, 1H), 4.95−5.0 (m, 2H), 5.25 (t, 1H, *J* = 9.6 Hz), 5.60 (d, 1H, *J* = 9.9 Hz), 7.2−7.5 (m, 13H, Ar−H), 7.88 (d, 2H, *J* = 7.7 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.93, 6.95, 20.64, 67.49, 67.86, 68.40, 68.46, 71.91, 72.57, 73.33, 73.94, 75.20, 79.30, 126.39, 127.35, 127.67, 127.85, 127.98, 128.09, 128.36, 128.54, 128.58, 129.10, 132.35, 132.68, 137.14, 137.91, 141.36, 153.60, 168.69; HRMS calcd for $C_{41}H_{52}INO_{13}SSiNa$ 976.190, found 976.187.

Ethylthio Sulfonamide Disaccharide 27. To a solution of iodo sulfonamide **26** (2.80 g, 2.93 mmol) in dry DMF (40 mL) at −40 °C was added EtSH (1.08 mL, 14.7 mmol), followed by dropwise addition of a solution of LHMDS (1.0 M solution in THF, 8.80 mL). The reaction mixture was stirred for 1 h, while it was allowed to warm to room temperature, quenched with a saturated solution of NH_4Cl (10 mL), and extracted with EtOAc (5 × 20 mL). The organic layer was washed with brine (15 mL), separated, dried ($MgSO_4$), filtered, and concentrated. The resulting crude material was redissolved in CH_2Cl_2 (50 mL) and treated with pyridine (1.0 mL), Ac_2O (1.0 mL), and the reaction mixture was stirred overnight. The organic layer was washed with a saturated solution of $CuSO_4$ (3 × 15 mL) and water (1 × 10 mL), and finally with a saturated solution of $NaHCO_3$ (2 × 15 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (1:1 EtOAc/hexanes) to provide **27** (2.38 g, 91%) as a syrup: $[\alpha]^{23}_D = -4.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3316, 2955, 2875, 1815, 1745, 1448, 1371, 1330, 1227, 1092, 897, 740 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.51 (q, 6H, *J* = 8.0 Hz, $-SiCH_2CH_3$), 0.88 (t, 9H, *J* = 7.9 Hz, $-SiCH_2CH_3$), 1.09 (t, 3H, *J* = 7.2 Hz, $-SCH_2CH_3$), 2.09 (s, 3H, $-COCH_3$), 2.44 (m, 2H, $-SCH_2CH_3$), 3.48 (br m, 1H, H-2), 3.83−3.70 (m, 7H), 3.89 (br t, 1H), 3.95 (br s, 1H), 4.43 (d, 1H, *J* = 5.4 Hz, H-1), 4.48 (br d, 2H, $-CH_2$Ar), 4.53 (d, 1H, *J* = 6.3 Hz, H-1′), 4.57 (s, 2H, $-CH_2$Ar), 4.75 (br t, 1H, *J* = 5.7 Hz, H-2′), 4.84 (br d, 1H, 9.9 Hz, $-NHSO_2Ph$), 7.20−7.40 and 7.40−7.60 (m, 13H, Ar−H), 7.97 (d, 2H, *J* = 7.2 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.28, 6.65, 14.56, 20.64, 20.89, 56.96, 67.64, 70.49, 70.52, 70.57, 71.14, 73.26, 73.72, 73.96, 74.99, 75.02, 76.88, 82.48, 97.83, 126.21, 127.30, 127.61, 127.78, 127.87, 128.29, 128.38, 128.62, 128.94, 132.17,

137.32, 137.94, 141.38, 153.27, 170.99; HRMS calcd for $C_{43}H_{57}NO_{13}S_2Si{\cdot}Na$ $(M + Na)^+$ 910.290, found 910.294.

Tetrasaccharide Diol 28. To a solution of a disaccharide **25** (0.100 g, 0.174 mmol) and thio donor **27** (0.308 g, 0.347 mmol) with 4 Å molecular sieves (1.0 g) in dry CH_2Cl_2 (8 mL) was added di-*tert*-butylpyridine (0.311 mL, 0.694 mmol). The suspension was cooled to −10 °C, treated with MeOTf (0.156 mL, 0.694 mmol), and stirred for 2 h. The reaction mixture was warmed to 0 °C, and stirred for 24 h. The reaction mixture was quenched with Et_3N (0.1 mL), stirred for an additional 3−5 min, diluted with EtOAc (25 mL), and filtered through a pad of silica gel. The filtrate was washed with a saturated solution of $NaHCO_3$ (2 × 10 mL), and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (35% EtOAc in hexanes) to provide 0.134 g (55%) of **28** tetrasaccharide as a syrup. Further elution (60% EtOAc in hexane) provided the regioisomer **29** in 3:1−2:1 ratio favoring the desired product: $[\alpha]^{23}_D = -28.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3491, 3029, 3874, 1815, 1753, 1647, 1453, 1370, 1221, 1160, 1064, 738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.38 (q, 6H, J = 8.0 Hz, $-SiCH_2CH_3$), 0.76 (t, 9H, J = 8.0 Hz, $-SiCH_2CH_3$), 1.97 (s, 3H, $-COCH_3$), 3.2−3.32 (m, 2H), 3.35−3.55 (m, 5H), 3.55−3.7 (m, 7H), 3.7−3.8 (m, 4H), 3.95 (dd, 1H, J = 4.6, 11.3 Hz), 4.0−4.12 (m, 2H), 4.18 (br s, 1H), 4.3−4.65 (m, 15H), 4.7−4.8 (m, 2H), 4.89 (t, 1H, J = 5.2 Hz), 5.31 (d, 1H, J = 8.4 Hz), 6.32 (d, 1H, J = 6.0 Hz, H-1), 7.1−7.5 (m, 28H, Ar−H), 7.85 (d, 2H, J = 7.4 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.47, 6.74, 20.62, 58.45, 67.89, 68.16, 68.91, 69.63, 70.01, 70.23, 70.70, 70.74, 72.95, 73.26, 73.32, 73.40, 73.43, 73.79, 74.38, 74.75, 74.81, 75.34, 76.60, 77.19, 82.21, 97.41, 100.41, 102.53, 102.84, 127.30, 127.44, 127.50, 127.56, 127.59, 127.62, 127.76, 127.82, 127.84, 127.96, 128.18, 128.24, 128.33, 128.38, 128.46, 128.83, 132.49, 137.32, 137.89, 138.70, 140.76, 144.51, 153.43, 168.94; HRMS calcd for $C_{74}H_{89}O_{22}SSiNa$ 1426.530, found 1426.526.

Tetrasaccharide 3′,4′-Diol 29: $[\alpha]^{23}_D = -40.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3469, 3030, 2873, 1814, 1254, 1451, 1369, 1222, 1063, 737 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.33 (q, 6H, J = 7.7 Hz, $-SiCH_2CH_3$), 0.73 (t, 9H, J = 7.5 Hz, $-SiCH_2CH_3$), 2.05 (s, 3H, $-COCH_3$), 3.11 (s, 1H, −OH), 3.30−3.50 (m, 8H), 3.60 (br m, 6H), 3.68−3.81 (m, 5H), 3.80 (dd, 1H, J = 4.9, 11.3 Hz), 4.02−4.12 (m, 2H), 4.14 (br s, 1H), 4.38−4.25 (m, 4H), 4.38−4.56 (m, 10H), 4.61 (dd, 2H, J = 6.0, 10.4 Hz), 4.61 (m, 1H), 4.70−4.77 (m, 2H), 4.89 (br t, 1H, J = 5.2 Hz), 5.49 (d, 1H, J = 8.5 Hz, $-NHSO_2Ph$), 6.31 (d, 1H, J = 6.4 Hz,m H-1), 7.10−7.30 (m, 25H, Ar−H), 7.32−7.45 (m, 3H, Ar−H), 7.87 (d, 2H, J = 6.8 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.43, 6.74, 20.74, 57.53, 67.68, 68.11, 69.54, 70.26, 70.30, 70.51, 70.70, 72.07, 72.72, 73.18, 73.34, 73.50, 73.72, 73.81, 73.85, 74.04, 74.16, 74.26, 74.38, 74.89, 70.05, 76.11, 76.59, 77.24, 96.95, 100.38, 103.09, 103.52, 127.37, 127.41, 127.46, 127.68, 127.82, 127.85, 127.99, 128.24, 128.29, 128.39, 128.41, 128.49, 128.97, 132.55, 137.37, 137.81, 137.89, 138.37, 138.65, 139.47, 144.53, 153.47, 169.69; LRMS calcd for $C_{74}H_{89}NO_{22}SSi{\cdot}Na$ 1426.5 $(M + Na)^+$, found 1426.6.

Alternative Synthesis of Tetrasaccharide Diol 28. To a solution of compound **25** (0.0983 g, 0.170 mmol) in dry benzene (90 mL) was added bis(tributyltin) oxide (0.0500 mL, 0.0935 mmol), and the resulting solution was distilled overnight with removal of water with a Dean−Stark trap. Thus formed tin ether was concentrated with a stream of dry N_2 and then further dried in vacuo. To a mixture of azeotropically dried (3 × 5 mL of benzene) compound **26** (0.0405 g, 0.0425 mmol) and freshly flame dried 4 Å molecular sieves (0.8 g) was added a solution of the tin ether in 1.8 mL of THF via cannula. Then the resulting suspension was cooled to −60 °C, and was treated with a solution of $AgBF_4$ (0.0337 g, 0.170 mmol) in 0.6 mL of THF via cannula. The reaction mixture was stirred for 2 days with exclusion of light while slowly being allowed to warm to room temperature. The reaction mixture was diluted with EtOAc (100 mL) and filtered through a pad of silica gel. The filtrate was washed with a saturated solution of $NaHCO_3$ (3 × 60 mL), and brine (1 × 60 mL). The organic layer was separated, dried (Na_2SO_4), filtered, concentrated, and submitted for chromatography (45% EtOAc in hexanes) to afford 0.0498 g (84%) of tetrasaccharide **28** as the only product. Further chromatographic separation (80% EtOAc in hexanes) afforded 0.0480 g of unused acceptor **25**.

Tetrasaccharide Pentaol 31. To a solution of tetrasaccharide **28** (0.37 g, 0.277 mmol) in MeOH (5 mL) was added K_2CO_3 (0.37 g), and the reaction mixture was stirred for 15 min. Then the solution was diluted with CH_2Cl_2 (100 mL), and filtered through a pad of silica gel, followed by a washing of the pad with EtOAc (100 mL). The filtrate was concentrated, and dried in vacuo without further purification to afford **31** (0.295 g, 85%) as a syrup: $[\alpha]^{23}_D = -18.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3469, 3030, 2873, 1648, 1496, 1452, 1328, 1092, 909, 737 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.31 (q, 6H, J = 6.4 Hz, $-SiCH_2CH_3$), 0.70 (t, 9H, J = 6.4 Hz, $-SiCH_2CH_3$), 2.49 (br s, 1H, −OH), 2.82 (br s, 1H, −OH), 3.16 (m, 1H, $-CHNHSO_2Ph$), 3.3−3.6 (m, 12H), 3.6−3.78 (m, 6H), 3.79 (br s, 2H), 3.8−3.85 (m, 3H), 3.92 (br d, 1H, J = 4.2 Hz), 4.0 (br t, 1H), 4.05−4.10 (m, 2H), 4.10−4.25 (m, 3H), 4.30−4.40 (m, 6H), 4.4−4.55 (m, 7H), 4.75 (dd, 1H, J = 2.7, 4.9 Hz), 4.9 (d, 1H, J = 4.2 Hz), 6.17 (d, 1H, 6.6 Hz, $-HNSO_2Ph$), 6.31 (d, 1H, J = 4.9 Hz, H-1), 7.0−7.4 (m, 23H, Ar−H), 7.80 (d, 2H, J = 6.0 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.30, 6.72, 57.88, 68.00, 68.78, 68.84, 69.20, 70.46, 70.86, 71.39, 71.99, 73.04, 73.14, 73.31, 73.40, 73.54, 73.79, 75.72, 76.01, 76.16, 81.44, 100.15, 101.85, 102.32, 102.60, 127.30, 127.45, 127.58, 127.62, 127.65, 127.73, 128.17, 128.21, 128.30, 128.33, 128.90, 132.52, 137.71, 137.87, 137.91, 138.10, 138.62, 140.18, 144.27; HRMS calcd for $C_{71}H_{89}NO_{20}SSiNa$ 1358.536 $(M + Na)^+$, found 1358.536.

Di-triethylsilylated Iodo Sulfonamide Disaccharide 32. To a solution of lactal **23** (2.50 g, 3.36 mmol) with 4 Å molecular sieves (3.0 g) and benzenesulfonamide (2.64 g, 3.36 mmol) was added at 0 °C a freshly prepared solution of $I(sym\text{-coll})_2ClO_4$ (5 equiv) in CH_2Cl_2. The reaction mixture was stirred at room temperature for 1 h, filtered through a pad of silica gel, and washed with a saturated solution of $Na_2S_2O_3$ (3 × 25 mL), $CuSO_4$ (5 × 25 mL), and water (2 × 10 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (5% EtOAc in CH_2Cl_2) to provide **32** (3.20 g, 92%) as a syrup: $[\alpha]^{23}_D = -19.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3258, 2953, 2875, 1806, 1788, 1453, 1331, 1105, 849, 745 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.57 and 0.64 (2q, 12H, J = 8.0 Hz, $-SiCH_2CH_3$), 0.90 and 0.95 (2t, 18H, J = 8.0 Hz, $-SiCH_2CH_3$), 3.39 (br m, 1H, H-2), 3.60−3.70 (m, 4H), 3.78−3.83 (br m, 2H), 4.05−4.17 (m, 3H), 4.34 (dd, 1H, J = 2.4, 8.7 Hz), 4.45 and 4.52 (2d, 2H, J = 12.0 Hz, $-CH_2Ar$), 4.55 (s, 2H, $-CH_2Ar$), 4.68 (d, 1H, J = 3.0 Hz), 4.89 (d, 1H, J = 8.6 Hz), 5.29 (t, 1H, J = 8.4 Hz), 5.47 (d, 1H, J = 9.6 Hz, $-NHSO_2Ph$), 7.2−7.5 (m, 13H, Ar−H), 7.89 (d, 2H, J = 7.6 Hz, Ar−H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 4.54, 4.94, 6.59, 6.95, 67.94, 68.12, 68.39, 68.63, 73.12, 73.31, 73.36, 73.90, 75.26, 75.33, 76.86, 79.66, 100.04, 127.40, 127.67, 127.76, 127.93, 128.01, 128.36, 128.51, 128.60, 132.39, 137.34, 138.02, 141.31, 154.01; HRMS calcd for $C_{45}H_{64}INO_{12}SSi_2{\cdot}Na$ 1848.260 $(M + Na)^+$, found 1848.263.

Di-triethysilylated Ethylthio Sulfonamide Disaccharide 33. To a solution of iodo sulfonamide **32** (2.70 g, 2.63 mmol) in dry DMF (40 mL) was added at −40 °C EtSH (0.584 mL, 7.89 mmol), followed by dropwise addition of a solution of LHMDS (1.0 M solution in THF, 7.89 mL). The reaction mixture was stirred for 1 h while being allowed to warm to room temperature. Then the solution was neutralized with a saturated solution of NH_4Cl (10 mL), and diluted with EtOAc (50 mL). The organic layer was washed with brine (5 mL), separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (3:7 EtOAc/hexanes) to provide **33** (2.30 g, 91%) as a syrup: $[\alpha]^{23}_D = -64.0°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3314, 2954, 2875, 1807, 1453, 1330, 1181, 1104, 739 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.50 (q, 6H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.624 (q, 6H, J = 7.6 Hz, $-SiCH_2CH_3$), 0.87 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.94 (t, 9H, J= 8.0 Hz, $-SiCH_2CH_3$), 1.11 (t, 3H, J = 7.4 Hz, $-SCH_2CH_3$), 2.48 (m, 2H,-SCH_2CH_3), 3.35 (m, 1H, H-2), 3.85−3.68 (m, 6H), 3.86 (br m, 1H), 3.97 (br t, 1H), 4.06 (br t, 1H, J = 6.6 Hz), 4.49 (s, 2H, $-CH_2Ar$), 4.57 (s, 2H, $-CH_2Ar$), 4.55 (m, 1H), 4.61 (d, 1H, J = 6.3 Hz, H-1), 4.67 (d, 1H, J = 4.0 Hz, H′-4), 4.87 (d, 1H, J = 8.8 Hz, H-1), 5.50 (d, 1H, J = 8.8 Hz, $-NHSO_2Ph$), 7.2−7.4 (m, 10H, Ar−H), 7.45−7.55 (m, 3H, Ar−H), 7.94 (d, 2H, J = 7.2 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.28, 4.45, 6.56, 6.71, 14.58, 25.64, 57.42, 67.82, 69.05, 69.68, 70.37, 71.80, 73.13, 73.66, 73.75, 76.45, 76.64, 77.05, 82.68, 100.94, 127.52,

127.57, 127.79, 127.83, 128.22, 128.35, 128.84, 132.25, 137.49, 138.01, 140.70; HRMS (FAB) calcd for $C_{47}H_{69}NO_{12}S_2Si_2Na$ 982.380, found 982.370.

Hexasaccharide Tetraol 35. To a solution of disaccharide **33** (0.200 g, 0.208 mmol) and tetrasaccharide **31** (0.278 g, 0.208 mmol) in CH_2Cl_2/Et_2O (1:2, 15 mL), with 4 Å molecular sieves (1.20 g) and di-*tert*-butylpyridine (0.180 mL, 0.778 mmol) at −10 °C, was added MeOTf (0.0880 mL, 0.778 mmol). The reaction mixture was stirred for 2 h, allowed to warm to 0 °C, and stirred for 24 h. Then the suspension was diluted with EtOAc (15 mL) and filtered through a pad of silica gel, and the filtrate was washed with a saturated solution of $NaHCO_3$ (2 × 10 mL). The organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (1:1 EtOAc/hexanes) to provide **35** (0.276 g, 60%) as a syrup: $[\alpha]^{23}_D = -23.0°$ (c, 1.0, $CHCl_3$); FTIR (film) 3490, 3030, 2875, 1807, 1649, 1453, 1330, 1093, 909, 743 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.25 (m, 6H, $-SiCH_2CH_3$), 0.37 (q, 6H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.71 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.76 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 0.86 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 2.46 (s, 1H, −OH), 2.52 (s, 1H, −OH), 3.15 (m, 1H, $-CHNHSO_2Ph$), 3.21 (m, 1H, $-CHNHSO_2Ph$), 3.28 (dd, 1H, J = 3.0, 9.2 Hz), 3.37−3.55 (m, 7H), 3.55−3.79 (m, 14H), 3.82 (br s, 2H), 3.89 (br s, 1H), 3.94−4.11 (m, 4H), 4.18 (br s, 1H), 4.28 (m, 1H), 4.33−4.40 (m, 3H), 4.41 (s, 2H, $-CH_2Ar$), 4.44−4.47 (m, 3H), 4.49 (s, 2H, $-CH_2Ar$), 4.52 (m, 1H), 4.54 (s, 2H, $-CH_2Ar$), 4.55−4.63 (m, 2H), 4.66 (dd, 2H, J = 3.9, 6.0 Hz), 4.74 (dd, 1H, J = 2.8, 6.1 Hz), 5.28 (d, 1H, J = 7.5 Hz, $-NHSO_2Ph$), 5.51 (d, 1H, J = 8.3 Hz, $-NHSO_2Ph$), 6.32 (d, 1H, J = 6.0 Hz, H-1), 7.10−7.55 (m, 41H, Ar−H), 7.83 (d, 2H, J = 7.4 Hz, Ar−H), 7.89 (d, 2H, J = 7.5 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.36, 4.44 (2C), 6.55, 6.67, 6.87, 58.52, 58.82, 67.61, 67.76, 67.82, 68.11, 68.71, 68.94, 69.07, 69.49, 69.75, 69.78, 69.92, 70.47, 70.73, 72.51, 72.92, 73.26, 73.31, 73.34, 73.37, 73.68, 73.85, 74.26, 74.61, 75.21, 75.27, 75.75, 75.90, 76.40, 77.10, 82.97, 83.60, 99.93, 100.49, 101.64, 102.74, 102.82, 103.08, 127.20, 127.38, 127.42, 127.49, 127.54, 127.62, 127.72, 127.76, 127.88, 128.10, 128.17, 128.26, 128.30, 128.40, 128.84, 128.97, 132.38, 132.71, 137.37, 137.57, 137.87, 137.90, 138.12, 138.18, 138.76, 139.9, 140.62, 144.45, 154.16; HRMS (FAB) calcd $C_{116}H_{152}N_2O_{32}S_2Si_3Na$ 2255.900 $(M + Na)^+$, found 2255.898.

Alternative Synthesis of Hexasaccharide Tetraol 35. To a solution of compound **31** (0.125 g, 0.0932 mmol) in dry benzene (90 mL) was added bis(tributyltin) oxide (0.0272 mL, 0.0513 mmol). The resulting solution was distilled overnight with removal of water with a Dean−Stark trap. Thus formed tin ether **34** was concentrated with a stream of dry N_2 and then further dried in vacuo. To a mixture of azeotropically dried (4 × 5 mL of benzene) compound **32** (0.0224 g, 0.0233 mmol) and freshly flame dried 4 Å molecular sieves (0.76 g) was added a solution of the tin ether **34** in 2.0 mL of THF via cannula. The resulting suspension was cooled to −60 °C, and was treated with a solution of $AgBF_4$ (0.0185 g, 0.0932 mmol) in 1.0 mL of THF via cannula. The reaction mixture was stirred for 4 days with exclusion of light while slowly being allowed to warm to room temperature. The reaction mixture was diluted with EtOAc (100 mL) and filtered through a pad of silica gel. The filtrate was washed with a saturated solution of $NaHCO_3$ (3 × 60 mL) and brine (1 × 60 mL), and the organic layer was separated, dried (Na_2SO_4), filtered, concentrated, and submitted for chromatography (40% EtOAc in hexanes) to provide 0.0321 g (62%) of hexasaccharide **35** as the only product. Further elution (80% EtOAc in hexanes) provided 0.0801 g of unused acceptor **31**.

Tetraacetylated Hexasaccharide 36. To a solution of hexasaccharide **35** (0.175 g, 0.0784 mmol) in dry CH_2Cl_2 (20 mL) were added pyridine (2 mL), Ac_2O (2 mL) and DMAP (catalytic). The reaction mixture was stirred for 24 h and washed with a saturated solution of $CuSO_4$ (3 × 10 mL) and $NaHCO_3$ (3 × 10 mL), and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (25% EtOAc in hexanes) to give rise to **36** (0.179 g, 95%) as a syrup: $[\alpha]^{23}_D = -30.0°$ (c 1.0, $CHCl_3$); FTIR (film) 3030, 2953, 1809, 1748, 1453, 1369, 1221, 1094, 738 (cm^{-1}); ^1H NMR ($CDCl_3$) δ 0.24 (m, 12H, $-SiCH_2CH_3$), 0.54 (q, 6H, J = 8.1 Hz, $-SiCH_2CH_3$), 0.68 (br t, 9H, J = 7.7 Hz, $-SiCH_2CH_3$), 0.70 (br t, 9H, J = 7.8 Hz, $-SiCH_2CH_3$), 0.87 (t, 9H, J = 7.9 Hz, $-SiCH_2CH_3$), 1.86 (s, 3H, $-COCH_3$), 1.90 (s, 3H, $-COCH_3$), 2.08 (s, 2H, $-COCH_3$), 2.15 (s, 3H, $-COCH_3$), 3.03 (br d, 1H, J = 7.7 Hz, $-CHNHSO_2Ph$), 3.2−3.4 (m, 8H), 3.4−3.85 (m, 30H), 3.85−4.2 (m, 8H), 4.20−4.6 (m, 29H), 4.75 (q, 1H, J = 3.1, 6.0 Hz), 4.8 (br d, 1H, J = 8.2 Hz), 4.88 (d, 1H, J = 3.5 Hz), 5.10 (m, 2H, J = 8.8 Hz), 5.26 (d, 1H, J = 2.5 Hz), 5.33 (d, 1H, J = 8.7 Hz, $-NHSO_2Ph$), 5.42 (d, 1H, J = 2.6 Hz), 5.90 (d, 1H, J = 10.8 Hz, $-NHSO_2Ph$), 6.31 (d, 1H, J = 6.0 Hz, H-1), 7.1−7.5 (m, 41H, Ar−H), 7.82 and 7.89 (2br m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$) δ 4.10, 4.14, 4.49, 6.52, 6.60, 6.64, 20.75, 20.81, 20.09, 21.46, 55.97, 56.73, 67.83, 68.41, 68.63, 68.80, 69.35, 69.82, 69.88, 70.12, 70.49, 71.09, 71.20, 71.71, 72.84, 72.95, 73.11, 73.38, 73.53, 73.60, 73.67, 73.74, 73.79, 74.10, 74.33, 74.40, 75.32, 75.78, 75.89, 76.18, 76.77, 77.20, 99.75, 100.15, 100.38, 100.53, 101.55, 102.17, 127.26, 127.34, 127.42, 127.47, 127.52, 127.58, 127.61, 127.62, 127.66, 127.73, 127.73, 127.80, 127.85, 128.14, 128.21, 128.26, 128.39, 128.41, 128.66, 128.99, 131.93, 132.60, 137.47, 137.66, 137.77, 137.92, 138.31, 138.43, 138.77, 139.96, 141.74, 144.48, 154.07, 169.44, 169.60, 169.64, 171.34; LRMS (ES) calcd for $C_{124}H_{160}N_2O_{36}S_2Si_3Na$ 2424.1 $(M + Na)^+$, found 2424.1.

Hexasaccharide Triol 37. To a solution of hexasaccharide **36** (0.175 g, 0.0725 mmol) in dry THF (5 mL) was added a solution of TBAF (1.0 M THF) and AcOH (1:1, 0.725 mL, 10 equiv). The solution was stirred at 35 °C for 24 h, diluted with EtOAc (10 mL), and washed with a saturated solution of $NaHCO_3$ (2 × 5 mL), and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (4:1 EtOAc/hexanes) to provide **37** (0.143 g, 93%) as a white glassy substance: $[\alpha]^{23}_D = -6.0°$ (c 1.0, $CHCl_3$); FTIR (film) 3472, 3028, 2870, 1805, 1745, 1369, 1225, 1161, 1069, 752 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.88, 1.92, 2.01, 2.02 (4s, 3H each, $-COCH_3$), 2.85 (br t, 1H, J = 8.2 Hz, $-CHNHSO_2Ph$), 3.02 (br q, 1H, J = 7.0 Hz, $-CHNHSO_2Ph$), 3.20 (dd, 1H, J= 7.6, 8.0 Hz), 3.27 (dd, 2H, J = 4.7, 10.0 Hz), 3.3−3.8 (m, 36H), 3.87 (br s, 2H), 4.03 (br d, 3H), 4.10 (br s, 1H), 4.2−4.65 (m, 33H), 4.66 (d, 1H, 5.1 Hz), 4.77 (q, 1H, J = 3.2 Hz), 5.01 (dd, 1H, J= 8.3, 9.7 Hz), 5.12 (dd, 1H, J = 8.2, 9.8 Hz), 5.25 (d, 1H, J = 3.2 Hz), 5.39 (d, 1H, J = 3.1 Hz), 6.32 (d, 1H, J = 6.1 Hz, H-1), 7.10−7.45 (m, 41H, Ar−H), 7.78 (m, 4H, Ar−H); ^{13}C NMR ($CDCl_3$) δ 20.69, 20.78, 21.18 (2C), 59.66 (2C), 67.79, 67.88, 68.12, 68.47, 69.42, 69.64, 70.13, 70.48, 71.14, 72.25, 72.70, 72.86, 73.11, 73.22, 73.38, 73.44, 73.50, 73.56, 73.71, 73.93, 74.29, 75.75, 77.20, 79.97, 80.25, 99.66, 100.99, 101.07, 101.18, 101.45, 101.55, 127.22, 127.29, 127.34, 127.63, 127.67, 127.73, 127.75, 127.80, 127.84, 128.00, 128.15, 128.26, 128.36, 128.41, 128.49, 128.61, 132.07, 132.20, 137.13, 137.34, 137.70, 137.71, 137.92, 138.21, 138.66, 140.96, 141.23, 144.43, 145.90, 170.04, 170.09, 170.15, 170.16; HRMS calcd for $C_{106}H_{118}N_2O_{36}S_2Na$ 2081.860, found 2081.676.

Nonasaccharide 39. To a solution of hexasaccharide **37** (0.140 g, 0.0680 mmol) and the known β-fluorofucose **38** (0.239 g, 0.530 mmol) in dry toluene (10 mL) with 4 Å molecular sieves at 0 °C was added 2,6-di-*tert*-butylpyridine (0.157 mL, 0.680 mmol) followed by a solution of $Sn(OTf)_2$ (0.228 g, 0.53 mmol) in THF (1 mL). The suspension was stirred for 36 h at room temperature and quenched with Et_3N (0.5 mL). The reaction mixture was stirred for an additional 3 min, diluted with EtOAc (25 mL), and filtered through a pad of silica gel. The organic layer was washed with a saturated solution of $NaHCO_3$ (2 × 10 mL), and separated, dried ($MgSO_4$), filtered, concentrated, and submitted for chromatography (1:1 EtOAc/hexanes) to provide **39** (0.135 g, 60%) as a syrup: $[\alpha]^{23}_D = -88.0°$ (c 1.0, $CHCl_3$); FTIR (film) 3029, 2869, 1817, 1745, 1722, 1452, 1270, 1096, 697 (cm^{-1}); ^1H NMR ($CDCl_3$) δ 0.87 (d, 3H, J = 6.2, $-CH_3$), 0.93 (d, 3H, J = 6.3 Hz, $-CH_3$), 1.07 (d, 3H, J = 6.4 Hz, $-CH_3$), 1.61 (s, 3H, $-COCH_3$), 1.80 (s, 3H, $-COCH_3$), 1.93 (s, 3H, $-COCH_3$), 1.98 (s, 3H, $-COCH_3$), 3.2−3.4 (m, 6H), 3.4−3.9 (m, 27H), 3.9−4.0 (m, 2H), 4.0−4.2 (m, 8H), 4.2−4.65 (m, 34H), 4.65−4.8 (m, 7H), 4.84 (d, 1H, J = 5.3 Hz), 4.89 (br t, 1H, J = 8.4 Hz), 5.05−5.15 (m, 2H), 5.28 (br s, 1H), 5.35 (d, 1H, J = 2.8 Hz), 5.40 (br s, 1H), 5.53 (br s, 3H), 5.65 (d, 1H, J= 5.6 Hz), 6.33 (d, 1H, J = 6.0 Hz, H-1), 7.0−7.3 (m, 68H, Ar−H), 7.3−7.45 (m, 9H, Ar−H), 7.45−7.57 (m, 3H), 7.65 (d, 2H, J = 7.6 Hz, Ar−H), 7.75 (d, 2H, J = 7.5 Hz, Ar−H), 7.99, 7.96, 7.94 (3d, 6H, J = 7.5 Hz, Ar−H); ^{13}C NMR ($CDCl_3$) δ 16.09, 16.13, 16.17, 20.38, 20.60, 21.16, 21.23, 56.42, 58.41, 64.97, 65.24, 65.59, 67.39, 67.40, 67.45, 67.86, 68.53, 68.71, 69.16, 69.35, 70.17, 70.25, 70.83, 70.99,

71.23, 71.25, 71.47, 71.50, 71.90, 72.55, 74.60, 73.14, 73.22, 73.39, 73.44, 73.49, 73.58, 73.66, 73.79, 73.81, 73.86, 74.72, 74.98, 75.37, 75.53, 75.71, 75.85, 76.10, 76.64, 76.94, 77.18, 96.01, 78.77, 79.40, 96.01, 97.38, 97.72, 98.67, 99.74, 100.00, 100.16, 100.24, 100.66, 127.11, 127.19, 127.25, 127.30, 127.37, 127.50, 127.60, 127.69, 127.79, 127.82, 127.88, 127.92, 128.02, 128.10, 128.12, 128.15, 128.29, 128.31, 128.39, 128.43, 128.99, 129.76, 129.84, 130.07, 132.27, 132.65, 132.90, 132.91, 133.18, 137.45, 137.48, 137.70, 137.73, 137.86, 137.90, 137.95, 138.03, 138.14, 138.17, 138.20, 138.26; LRMS calcd for $C_{187}H_{196}N_2O_{51}S_2 \cdot 2Na$ 3395.2 $(M + 2Na)^{2+}$, found 3395.2.

Nonasaccharide Thioglycoside 41. To a solution of a nonasaccharide **39** (0.0502 g, 0.0150 mmol) in dry CH_2Cl_2 (1 mL) with 4 Å molecular sieves (0.10 g) was added a solution of dimethyldioxirane in acetone (ca. 0.08 M, 3 mL). The solution was stirred for 45 min, and the solvent was removed with a stream of dry N_2, and further dried in vacuo (10 min). The resultant crude epoxide was dissolved in CH_2Cl_2 (1 mL), and was treated with EtSH (1 mL) and TFAA (0.005 mL) at −78 °C. The reaction mixture was stirred for 6 h while warming to room temperature. Volatiles were evaporated with a stream of dry N_2 and in vacuo. The crude mixture was dissolved in CH_2Cl_2 (1 mL) and then treated with acetic anhydride (0.5 mL) and pyridine (0.5 mL). After 24 h of stirring, the reaction mixture was concentrated under reduced pressure and purified by flash chromatography (2:3 EtOAc/hexanes) to afford thioglycoside **41** (0.0302 g, 60%) as a syrup: $[\alpha]^{23}_D = -0.030°$ (*c* 1.0, $CHCl_3$); FTIR (film) 3064, 2875, 1808, 1744, 1720, 1453, 1369, 1250, 1090, 730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.85 (d, 3H, $J = 6.0$ Hz, $-CH_3$), 0.93 (d, 3H, $J = 6.1$ Hz, $-CH_3$), 1.07 (d, 3H, $J = 6.2$ Hz, $-CH_3$), 1.18 (dt, 3H, $J = 1.5$ Hz, $-SCH_2CH_3$), 1.67 (s, 3H, $-COCH_3$), 1.81 (s, 3H, $-COCH_3$), 1.92 (s, 3H, $-COCH_3$), 1.97 (s, 3H, $-COCH_3$), 2.05 (s, 3H, $-COCH_3$), 2.62 (m, 2H, $-SCH_2CH_3$), 3.10 (br m, 1H), 3.2−3.4 (m, 7H), 3.4−3.75 (m, 24H) 3.8 (m, 3H), 3.82 (dd, 2H), 3.85−4.0 (m, 4H), 4.0−4.2 (m, 4H), 4.2−4.5 (m, 26H), 4.5−4.63 (m, 1H), 4.63−4.8 (m, 8H), 4.8−5.0 (m, 5H), 5.11 (d&m, 2H), 5.21 (d, 1H), 5.35 (d, 1H), 5.40 (br s, 1H), 5.54 (br s, 2H), 5.64 (br s, 1H), 7.0−8.2 (m, 90H, Ar−H); LRMS calcd for $C_{191}H_{204}N_2O_{53}S_3 \cdot NH_4$ 3369.2 $(M + NH_4)^+$, found 3469.0.

Azide 42. To a solution of thioglycoside **41** (0.0290 g, 0.00860 mmol), azidohyrin **40** (0.0357 g, 10.0 equiv) at 0 °C in dry Et_2O/CH_2Cl_2 (2:1, 1.5 mL), and 4 Å molecular sieves (0.10 g) was added MeOTf (0.00389 mL, 4 equiv). The reaction mixture was stirred while warming to room temperature. After 24 h of stirring, the suspension was quenched with Et_3N (0.020 mL), diluted with EtOAc (5 mL), filtered through a pad of silica gel, and washed with a saturated solution of $NaHCO_3$ (2 × 5 mL), and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and submitted for column chromatography (1:1 EtOAc/hexanes) to provide azide **42** (0.0181 g, 55%) as a syrup: $[\alpha]^{23}_D = -17.4°$ (*c* 0.7, $CHCl_3$); FTIR (film) 3030, 2923, 2100, 1814, 1744, 1602, 1451, 1368, 1269, 1222, 1096, 743 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.80 (br t, 3H, $-CH_3$), 0.85 (d, 3H, $J = 6.3$ Hz, $-CH_3$), 0.93 (d, 3H, $J = 6.3$ Hz, $-CH_3$), 1.07 (d, 3H, $J = 6.4$ Hz, $-CH_3$), 1.18 (br s, 23H, aliphatic $-CH_2$), 1.33 (br m, 2H) 1.52 (s, 3H, $-COCH_3$), 1.81 (s, 3H, $-COCH_3$), 1.94 (s, 3H, $-COCH_3$), 2.02 (s, 6H, 2-$COCH_3$), 3.20 (m, 2H), 3.3 (m, 5H), 3.38−3.43 (m, 5H), 3.5−3.75 (m, 20H), 3.75−3.83 (br d, 2H), 3.85 (br q, 2H, $J = 5.8$, 8.8 Hz), 3.9−4.3 (m,4H), 4.3−4.54 (m, 3H), 4.54−4.63 (m, 7H), 4.63−4.82 (m,7H), 4.83 (d, 1H, $J = 5.0$ Hz), 4.89 (t, 1H, $J = 9.6$ Hz), 5.10 (d, 1H, $J = 3.5$ Hz), 5.12 (m, 1H), 5.26 (br s, 1H), 5.34 (m, 1H), 5.39 (br d, 2H, $J = 8.2$ Hz), 5.47 (d, 1H, $J = 8.2$ Hz), 5.53 (br s, 2H), 5.60 (d, 1H, $J = 5.5$ Hz), 5.69 (dt, 1H, $J = 6.6$, 14.7 Hz), 7.0−7.3 (m, 80H, Ar−H), 7.33−7.43 (m, 8H), 7.5 (m, 4H), 7.65 (d, 2H, $J = 7.6$ Hz, Ar−H), 7.78 (d, 2H, $J = 7.5$ Ar-H), 7.97 (m, 6H, Ar−H); LMRS calcd for $C_{214}H_{239}N_5O_{55}S_2 \cdot 2NH_4$ 3858.5 $(M + 2NH_4)^{2+}$, found 3859.0.

Protected KH-1 Antigen 43. To a solution of azide **42** (0.0181 g, 0.00473 mmol) in EtOAc (3 mL) were added Lindlar's catalyst (0.050 g) and palmitic anhydride (0.0102 g, 0.0200 mmol), and the reaction mixture was stirred at room temperature under a H_2 atmosphere for 24 h. The reaction mixture was filtered through a pad of silica gel, rinsed with EtOAc (20 mL), concentrated, and submitted for chromatography (1:1 EtOAc/hexanes) to provide amide **43** (0.0165 g, 85%) as a syrup: $[\alpha]^{23}_D = -36.0°$ (*c* 0.5, $CHCl_3$); FTIR (film) 3025, 2923, 1814, 1745, 1657, 1451, 1367, 1266, 1095, 742 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.80 (m, 6H, $-CH_3$), 0.85 (d, 3H, $J = 6.0$ Hz, $-CH_3$), 0.93 (d, 3H, $J = 6.4$ Hz, $-CH_3$), 1.07 (d, 3H, $J = 6.4$ Hz, $-CH_3$), 1.18 (br s, 48H, aliphatic $-CH_2$), 1.6 (m, 4H), 1.84 (s, 6H, 2-$COCH_3$), 2.0 (s, 3H, $-COCH_3$), 2.08 (s, 6H, $-COCH_3$) 3.2 (br m, 1H), 3.3 (br m, 6H), 3.3−3.75 (m, 27H), 3.78 (m, 3H), 3.85 (m, 2H), 3.95 (m, 3H), 4.2 (dd,4H), 4.15−4.5 (m, 23H), 4.5−4.75 (m, 9H), 4.75−4.8 (m, 4H), 4.8−4.95 (m, 3H), 5.1 (br s, 2H), 5.26 (m, 2H), 5.57 (m, 2H), 5.59 (m, 2H), 7.00−7.35 (m, 79H, Ar−H), 7.4 (m, 8H, Ar−H), 7.5 (m, 3H, Ar−H), 7.65 (d, 2H), 7.75 (2d, 2H), 7.96 (m, 6H); ^{13}C NMR (anomeric carbons) δ 98.06, 100.11 (2C), 100.47, 101.28, 102.74, 102.89, 102.97, 103.16; LRMS calcd for $C_{230}H_{271}N_3O_{56}S_2 \cdot NH_4$ 4052.8 $(M + NH_4)^+$, found 4052.0.

KH-1 Antigen (1). To a blue solution of sodium (0.018 g) in liquid ammonia (5 mL) under N_2 at −78 °C was added a solution of protected KH-1 derivative **43** (0.0202 g, 0.00500 mmol) in dry THF (1 mL). After 45 min of reflux at −78 °C, the reaction mixture was quenched with absolute MeOH (5 mL). Most of the ammonia was removed with a stream of dry nitrogen, and the solution was diluted with MeOH (5 mL) and stirred overnight. The solution was neutralized with $Et_3N \cdot HCl$, stirred for an additional 15 min, and dried with a stream of dry nitrogen. The crude material obtained was then suspended in DMF (1.0 mL), THF (1.0 mL), and Et_3N (1.0 mL) and treated with Ac_2O (1 mL) and a catalytic amount of DMAP. The reaction mixture was stirred for 24 h. The solution was concentrated, passed through a pad of silica gel with the help of EtOAc, and again concentrated. The syrup obtained was dissolved in MeOH (5 mL), treated with MeONa (0.005 mL), neutralized with Dowex 50-X8 after 24 h, filtered, and concentrated to give 0.00711 g (70%) of KH-1 antigen (**1**). An analytical sample was prepared by RP column chromatography, eluting with water−5% methanolic water, followed by lyophilization to deliver **1** as a white foam: ^1H NMR (DMSO) δ 0.95 (m, 3H), 1.1−1.35 (3d, 9H, $-CH_3$), 1.38 (br m, multiple protons, aliphatic $-CH_2$), 1.5 (m, 9H), 1.85 (s, 6H, $-CHNHCOCH_3$), 1.9 (m, 2H), 2.0−2.20 (m, 6 H), 3.0−4.0 (m, multiple protons), 4.01 (q, 1H, $J = 6.5$ Hz), 4.17 (d, 1H, $J = 7.5$ Hz), 4.27 (br d, 1H), 4.34 (br m, 1H), 4.40 (d, 1H, $J = 7.0$ Hz), 4.59 (m, 1H), 4.65 (d, 1H, $J = 8.0$ Hz), 4.67 (d, 1H, $J = 4.0$ Hz), 4.72 (d, 1H, $J = 7.0$ Hz), 4.73 (d, 1H, $J = 7.5$ Hz), 4.87 (d, 1H, $J = 3.5$ Hz), 4.97 (d, 1H, $J = 3.5$ Hz), 5.37 (dd, 1H, $J = 7.0$ Hz, 15.5 Hz), 5.55 (dt, 1H, 6.5 Hz, 15.0 Hz); ^{13}C NMR (DMSO, anomeric carbons) δ 101.79, 102.40, 103.60, 103.78, 104.51 (2C), 105.18, 106.70, 106.77; HRMS (FAB) calcd for $C_{92}H_{163}N_3O_{45} \cdot Na$ 2053.046 $(M + Na)^+$, found 2053.049.

Allyl Glycoside 44. To a solution of sodium (0.060 g) in liquid ammonia (8 mL) under N_2 at −78 °C was added a solution of nonasaccharide glycal **39** (0.0469 g, 0.0140 mmol) in dry THF (2 mL). After 45 min of reflux at −78 °C, the reaction mixture was quenched with absolute MeOH (5 mL). Most of the ammonia was removed with a stream of dry nitrogen and then diluted with MeOH (5 mL) and stirred overnight. The basic solution was neutralized with Dowex 50-X8 (0.557 g), and the mixture was stirred for an additional 15 min, and filtered. The resins were washed with a saturated solution of NH_3 in MeOH (4 × 50 mL), and all filtrates were combined and dried with a stream of dry nitrogen. The crude material obtained was then suspended in DMF (1.0 mL), THF (1.0 mL), and Et_3N (1.0 mL), treated with Ac_2O (1 mL) and a catalytic amount of DMAP, and stirred for 24 h. The solution was concentrated, passed through a pad of silica gel with the help of EtOAc, and concentrated. The syrup obtained was dissolved in CH_2Cl_2 and then at 0 °C under N_2 treated with dimethyldioxirane solution in acetone (ca. 0.08 M, 6 mL) and stirred for 45 min. Solvent was removed with a stream of N_2. The resultant syrup (0.0400 g) was redissolved in allyl alcohol (5 mL). After 24 h, excess allyl alcohol was evaporated, and the crude syrup was dissolved in MeOH (5 mL) and treated with MeONa (25% in MeOH, 0.060 mL). After 24 h, the mixture was neutralized with Dowex 50-X8, filtered, and concentrated to provide allyl glycoside **44** (0.0130 g, 60%). An analytical sample was prepared by RP column chromatography, eluting with water−5% methanolic water, followed by lyophilization to obtain a white foam: $[\alpha]^{23}_D = -58.0°$ (*c* 0.6, H_2O); ^1H NMR (D_2O) δ 1.12 (d, 3H, $J = 6.5$ Hz, $-CH_3$), 1.21 (d, 3H, $J = 7.0$ Hz, $-CH_3$), 1.24 (d, 3H, $J = 6.5$ Hz, $-CH_3$), 2.00 (1.35 (3d, 9H, $-CH_3$), 2.0 (s, 6H, $-COCH_3$), 3.31 (br t, 1H, $-CHNHAc$), 3.4−4.0 (m, multiple protons), 4.06 (d, 1H, $J = 3.0$ Hz), 4.13 (d, 1H, $J = 3.0$ Hz), 4.22 (m, 2H), 4.41 (d, 1H, $J = 8.0$ Hz,

anomeric), 4.49 (d, 1H, J = 8.0 Hz, anomeric), 4.51 (d, 1H, J = 8.5 Hz, anomeric), 4.69 (d, 2H, J = 8.5 Hz, 2 anomeric), 4.79 (q, 1H, J = 7.0 Hz), 4.86 (q, 1H, J = 7.0 Hz), 5.09 (d, 1H, J = 4.0 Hz, anomeric), 5.10 (d, 1H, J = 4.0 Hz, anomeric), 5.25 (d, 1H, J = 3.0 Hz, anomeric) 5.36 (d, 1H, J = 17 Hz, $-CH=CH_2$), 5.95 (m, 1H, $-CH=CH_2$); ^{13}C NMR (D_2O, anomeric carbons) δ 101.31 (2C), 102.20, 102.91, 103.79, 104.50, 105.23 (2C), 105.70; HRMS (FAB) calcd for $C_{61}H_{102}N_2O_{43}$·Na 1573.575 $(M + Na)^+$, found 1573.568.

Synthesis of KH-1 Aldehyde. Ozone gas was bubbled through a solution of 0.004 g of KH-1 allyl glycoside **50** in 5 mL of methanol for 10 min while the solution was stirred vigorously at −78 °C. The excess ozone was then displaced with nitrogen over a period of 5 min. To the solution was added 0.10 mL of methyl sulfide. The reaction mixture was stirred for 2 h at room temperature.

Conjugation of KH-1 Aldehyde with KLH. To a solution of 0.002 g of KH-1 aldehyde and 0.004 g of keyhole lympet hemocyanin (KLH) in 1.0 mL of 0.1 M phosphate-buffered saline (PBS), pH 7.2, was added 0.0020 g of sodium cyanoborohydride, and the mixture was incubated under gentle agitation at 37 °C. After 16 h, an additional 0.001 g of sodium cyanoborohydride was added, and the incubation was continued for another 22 h. The unreacted KH-1 aldehyde was removed completely with multiple washes using a Amicon Centriprep with molecular weight cutoff value of 30 000, with 6−7 changes of PBS at 4 °C. The epitope ratio was determined by estimating protein content by BioRad dye binding protein assay and carbohydrate by a HPAEC-PAD assay. The epitope ratio was 141:1.

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Supporting Information Available: Experimental procedures for **30**, **45**, **46**, **47**, **48**, **49**, and **50** and NMR spectra (^1H for **1**, **16**, **19**, **20**−**33**, **35**−**37**, **39**, and **41**−**50**, ^{13}C for **16**, **19**, **20**−**33**, **35**−**37**, **39**, and **45**−**49**, and HMQC for **1**, **43**, **44**, and **50**) (67 pages). See any current masthead page for ordering information and Web access instructions.

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A Fully Synthetic Globo H Carbohydrate Vaccine Induces a Focused Humoral Response in Prostate Cancer Patients: A Proof of Principle**

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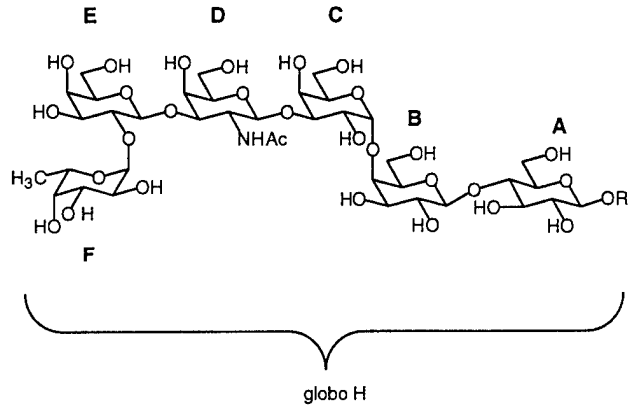
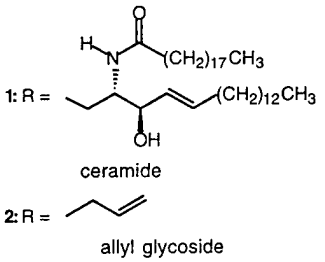
The enlistment of the formidable resources of the human immune system against cancerous lesions has been a long-standing vision in medicine.[1, 2] Ideally, a vaccine containing a particular tumor-associated antigen or a range of cell-surface antigens, presented in an effective immunostimulatory context, would trigger active immunity against cancer cells expressing counterpart structures on their surfaces.[3] The focus of our investigation is the development of a vaccine strategy that would be active in an adjuvant or minimum disease setting, providing enhanced protection against micrometastasis in a context where the primary tumor has been eliminated through surgery, radiation, or chemotherapy. The primary targets of our studies have been carbohydrate-based antigens such as glycolipids, or glycoproteins (including mucins), expressed on the accessible surfaces of tumor cells.[4, 5]

In this particular study, we have concentrated on the globo H tumor antigen. This antigen was first identified chemically from breast tumor extracts by Hakomori et al.[6] It was immunocharacterized by Colnaghi et al. (mAb MBr1),[7] and more recently by Lloyd et al. (mAb VK-9)[8] (mAb = monoclonal antibody). By the criteria of immunohistology, globo H was identified on a number of human cancers (including prostate and breast cancer) and in a restricted number of normal epithelial tissues.[9]

To proceed in a productive fashion, it was first necessary for us to accomplish a total synthesis of the globo H antigen (**1**) and properly conjugated immunogenic variants thereof, in adequate quantities for preclinical studies. These goals were





3: R = $(CH_2)_2$ $(Lys)_n$ - KLH

4: delete ring A from **2**

5: delete rings A and B from **2**

6: ring C β-glycoside of **2**

7: ring D α-glycoside of **2**

8: defucosyl of **1**

achieved by drawing heavily on the principles of glycal assembly (Scheme 1).[10, 11] The allyl group in the fully synthetic glycoside (**2**) was used as a spacer element, and as a point of chemical access for conjugating the hexasaccharide antigen of globo H to the carrier protein keyhole limpet hemocyanin (KLH; see structure **3**).

Following favorable serological and cell-surface reactivity in the vaccination of mice with construct **3**,[12] and after successful scale-ups in the total synthesis, a clinical trial using fully synthetic globo H vaccines in prostate cancer patients was launched. The vaccine construct **3**, in conjunction with the QS-21 adjuvant, had proven to be particularly immunogenic in the murine setting for eliciting globo H-specific responses.[12]

It should be emphasized that in progressing from a murine to a human setting for the vaccination, two potential risks had to be faced. Human sera and cell-surface glycoproteins present related structures (such as Lewis blood group determinants and, indeed, low levels of globosides). Hence, in the human clinical setting, there were potential issues of immunotolerance or possibly autoimmune responses to be addressed which were not pertinent to the earlier experiments with mice.

Here we report on encouraging early results from our clinical investigation using conjugate system **3** in tandem with QS-21. Five patients with progressive and recurrent prostate cancer received the conjugate vaccine, containing 30 μg of

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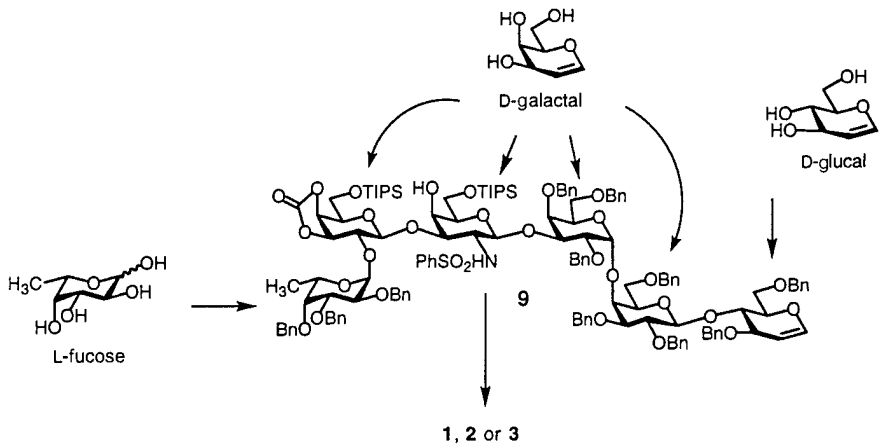

Scheme 1. The total synthesis of globo H hexasaccharide showing the logic of glycal assembly. Bn = benzyl, TIPS = triisopropylsilyl.

globo H plus QS-21, according to defined clinical protocols. Their sera were submitted for detailed analyses and evaluation. By ELISA (enzyme-linked immunosorbent assay), no detectable IgM or IgG antibodies against synthetic globo H were present prior to vaccination (Figure 1). The pre- and post-vaccination IgM and IgG ELISA titers against globo H-ceramide in sera from all five patients are shown in Figure 1. Subsequent to vaccination, all five patients produced a strong IgM response, while two concurrently generated a high IgG response. The specificity of these antibodies for globo H (derived from synthesis) or for globo H in prostate cancer cell extract from tumor or biopsy, as well as breast cancer biopsy specimens, was analyzed by immune thin layer chromatography (ITLC, Figure 2; results of all five patients are summarized in Table 1). Though the vaccines were based on a synthetic globo H-protein conjugate, the postvaccination sera recognized both synthetic and tumor-derived globo H-ceramide. A similar finding has been described for the VK-9 anti-

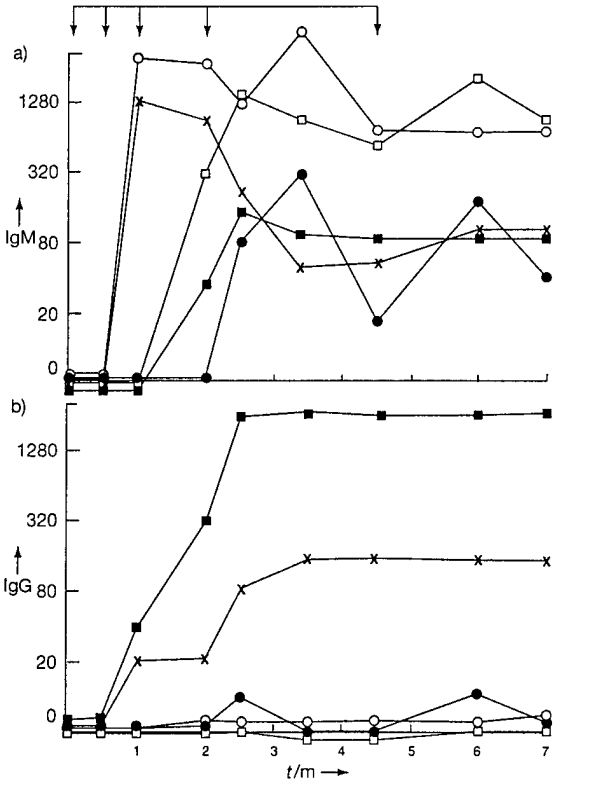

Figure 1. Time course of the induction of antibodies in five patients immunized with globo H-KLH conjugate and QS-21: a) IgM titer, b) IgG titer. ●: patient 1; × : patient 2; ○: patient 3; □: patient 4; ■: patient 5. The y axis shows the reciprocal titer against globo H by ELISA, and the x axis the time t in months. The arrows pointing down indicate vaccinations with globo H-KLH (30 μg) plus QS-21.

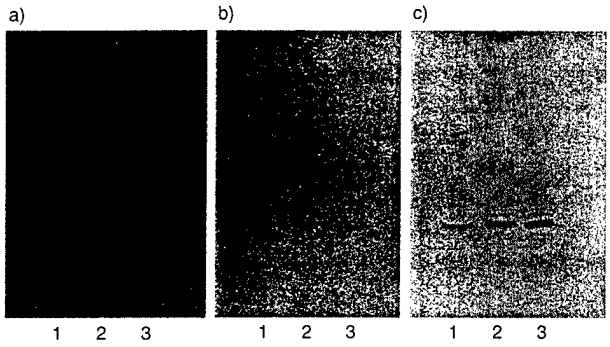

Figure 2. Immune thin-layer chromatography (ITLC) with synthetic and natural antigens and sera from patient 2 vaccinated with globo H-KLH conjugate. In each case, antigen from prostate cancer extract, antigen from breast cancer extract, and synthetic globo-H antigen, is applied in lanes 1, 2, and 3, respectively. Detection was achieved a) with a solution of the antibody mAbMBr1, b) with prevaccination sera, and c) with post vaccination sera.

body.[8, 10] By contrast, the sera failed to react with melanoma biopsy specimen extracts, which contain various glycolipids but are globo H negative.

Two different types of ELISA inhibition assays were carried out to determine the specificity of the anti-globo H antibodies in the immunized patients: 1) inhibition of reactivity against globo H-ceramide with structurally related antigens previously obtained by total synthesis in our laboratory[10, 12] as well as structurally unrelated antigens as controls; and 2) inhibition (absorption) by globo H-positive and -negative cell lines. The results of these studies (shown in Figure 3a, b) demonstrate that globo H-ceramide inhibits anti-globo H reactivity more efficiently than any of the other structurally related congeners obtained through synthesis. For instance, globo H allyl glycoside **2** at a concentration of 500 μM inhibited 40% of the anti-globo H antibody activity while globo H-ceramide **1** at the same concentration inhibited 90% of the reactivity. This finding is particularly interesting in that the immunizing antigen (globo H-KLH) lacked the ceramide moiety. All truncated oligosaccharide isomers of globo H previously prepared by synthesis[13] were also recognized, though less so than **1**. For example, SSEA-3 (**8**; SSEA = stage-specific embryonic antigen), which lacks the fucose residue, also

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Table 1. Summary of immune thin layer chromatography with synthetic and natural antigens and sera from five patients vaccinated with globo H–KLH conjugate.[a]

Patient	globo H–ceramide		Prostate CA extract		Breast CA extract		Melanoma extract	
	pre	post	pre	post	pre	post	pre	post
1	–	+++	–	+	–	+	–	–
2	–	+++	–	++	–	++	–	–
3	–	++	–	++	–	++	–	–
4	–	++	–	++	–	++	–	–
5	–	+++	–	++	–	++	–	–
mAb Mbr1[b]	++++		+++		+++		–	

[a] CA = carcinoma; pre = before vaccination, post = after vaccination. [b] Reference antibody (positive control).

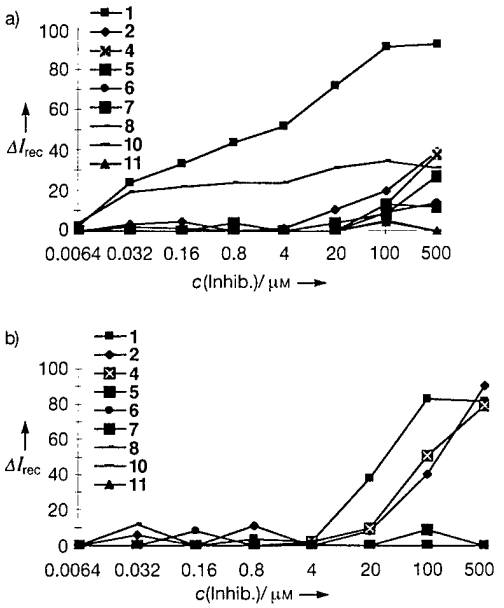

Figure 3. Analysis of the specificity of anti-globo H antiserum by inhibition assays. ELISA reactivity of serum with globo H-ceramide inhibited with compounds **1**, **2**, **4**–**8**, **10**, **11**: a) IgM antibody response, b) IgG antibody response. ΔI_{rec} = inhibition (%), *c* (Inhib.) = concentration of the inhibitor.

inhibited 30% of the antibody reactivity (Figure 3a). As a control, the unrelated glycolipid GD3 **10** containing the ceramide chain, but otherwise lacking any resemblance to the carbohydrate sector of the globo H and synthetic Le^y–allyl glycoside **11**, showed no inhibition.

GD3 = NeuAc-α(2→8)-NeuAc-(2→3)-Gal-β(1→4)Glc-β(1–1′)-Cer **10**

Le^y = Fuc-α(1→2)-Gal-β(1→4)-(Fuc-α(1→3))-GlcNAc-β(1→3)-Gal-β-*O*-Allyl **11**

The IgG response, however, was found to be quite different. In the two antisera demonstrating IgG ELISA reactivity, synthetic hexasaccharides **1** and **2**, as well as a pentasaccharide analogue (see compound **4**) derived from the nonreducing end of the molecule effectively inhibited binding in this assay (Figure 3b). No inhibition was seen with SSEA-3 **8**, which lacks the fucose residue or the tetrasaccharide **5**, which lacks the lactose moiety. Thus, IgG antibodies from both antisera appear to mainly recognize an epitope area encompassing five nonreducing terminal carbohydrate units (see segments B–F).

The lack of recognition of Lewis^Y antigen **11** is particularly noteworthy since many of the constituent building blocks in **1** are also present in **11**. Clearly, the specificity for **1** arises from the difference in the structural and stereochemical connectivity of the antigenic subunits. The general message which comes through from the inhibitory characteristics of our fully synthetic various terminal probe structures is that of a polyclonal, but focused, response against various portions of globo H. The results show that a fucosylated tetra- or pentasaccharide structure is required for an optimal anti-globo H response.

Encouraged by these results, we evaluated whether antibodies elicited by synthetic vaccine **3** recognize the globo H antigen in its natural context, that is, the cell surface. This type of challenge is a crucial milestone in the progression of antitumor vaccines. Two assays were developed to measure the cell surface binding. In the first assay, sera were mixed with globo H-positive (MCF-7) and globo H-negative cell lines (SK-MEL-28). More than 50% of the ELISA reactivity against globo H–ceramide was lost following incubation with MCF-7 cells in all patients. No decrease in binding activity was observed following incubation with globo H-negative SK-MEL-28 melanoma cells. Comparable results were obtained in control experiments with mAb VK-9.

Furthermore, the cell-surface reactivity of anti-globo H antibodies was tested by flow cytometry (Figure 4). As judged by this assay, sera before vaccination showed very low

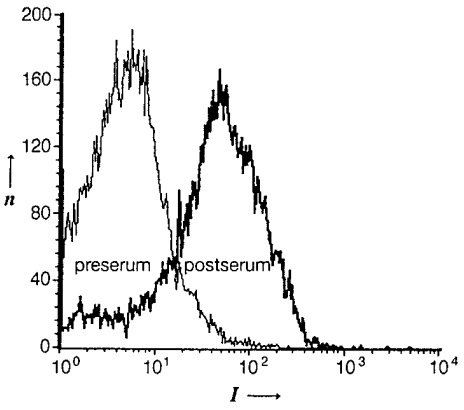

Figure 4. Reactivity of representative pre- and postvaccination sera with MCF-7 cells by flow cytometry. *n* = relative MCF-7 cell number (counts). *I* = fluorescence intensity.

reactivities with cell surfaces. However, sera drawn after the fourth vaccination showed an increase of IgM reactivity with MCF-7 breast cancer cells ranging from 11 to 97%. In a

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similar vein, the increase of the IgG antibody cell surface reactivites ranged from 15 to 35%.

As the last element of the serologic evaluation following vaccination, we also tested the anti-globo H sera for their ability to mediate complement-dependent cytotoxicity (CDC). Three of the five postvaccination sera showed strong CDC to MCF-7 cells. In control experiments, we found that 1) the same sera in the absence of complement, 2) complement without sera, and 3) prevaccination sera with complement failed to exhibit CDC under the same conditions.

In summary, our globo H glycoconjugate based vaccine (**3**) induces strong and well targeted humoral immune responses in patients. The resultant antibodies not only recognize the synthetic antigens (**1** and **2**) but also globo H-positive tumor biopsy extracts and tumor tissues. The antisera following vaccination successfully mediated complement-induced lysis of relevant cancer cells.

We note that, in a recent study in melanoma, antibodies induced in the setting of microscopic disease seem to be associated with decreased tumor outgrowth and appear to favor longer patient survival times.[14] In this context, the serological findings described here through vaccination of prostate cancer patients with the globo H vaccine portend a clinical advantage.

A formal full clinical report on this trial and a related globo H-directed breast cancer trial, conducted in larger patient populations, will be presented separately. Following the important "proof of principle" findings described herein, and other clinical indications associated with tumor-related complex carbohydrate antigens to be described soon, new trials in prostate and breast cancer patients using totally synthetic carbohydrate vaccines are in various stages of development.

Experimental Section

Vaccine preparation and clinical protocol:

The globo H–KLH conjugate was prepared in a manner similar to sialyl Tn cluster–KLH conjugate, as previously described.[15] Patients with progressive prostate cancer that had a minimum of three rising prostate-specific antigen (PSA) values were vaccinated with globo H-KLH vaccine containing 30 μg of globo H and 100 μg of QS-21. Three vaccinations were administered subcutaneously at one week intervals. Two additional vaccinations were administered at week 7 and 19. Peripheral blood (20–30 mL) was drawn immediately before each vaccination, and two weeks after the fourth and fifth vaccinations. The sera obtained from prevaccination and two weeks after the third, fourth, and fifth vaccinations of all patients were tested for antibodies against globo H–ceramide, truncated globo H analogues, tumor extracts, and the globo H-positive MCF-7 cell-line.

Serological analysis:

ELISA: ELISAs were performed as described previously.[8, 16] ELISA plates were coated with globo H–ceramide at 0.1 μg per well. Serially diluted patient serum was added to wells of the coated plates, and antibody titer was defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal patient sera.

Immune thin layer chromatography (ITLC): Immune staining of synthetic globo H–ceramide, and the neutral glycolipid extract obtained from breast and prostate cancers with patient sera or mAb MBr1, was performed after separation on HPTLC silica gel glass plates as previously described.[8, 16] Patient sera diluted appropriately in phosphate-buffered saline (PBS), and anti-human IgG or IgM antibodies conjugated with horseradish peroxidase (Biosource International, Camarillo, CA) at 1:200 dilution were used.

Inhibition assay: Antisera at appropriate dilution or mAb VK-9 at 0.1 $\mu g\,mL^{-1}$ were mixed with various concentrations of structurally related and unrelated carbohydrate antigens. The mixture was incubated overnight at 4 °C, and used in ELISA assays as described above. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum.

ELISAs were also performed with sera that had been inhibited (absorbed) by incubation with MCF-7 or SK-MEL-28 cells. For this assay 17×10^6 cells were incubated with sera for 1 h, and the cells removed by centrifugation. ELISA was performed as described above.

Fluorescence-activated cell sorter (FACS) assay: FACS analyses were performed as previously described[8, 16] using FACS Scan (Becton-Dickinson, CA). Cells from the globo H-positive breast cancer cell line MCF-7 or the globo H-negative melanoma cell line SK-Mel-28 served as targets. An aliquot of 20 μL of diluted (1:20) antisera or mAb MBr1 and 20 μL of 1:30 goat anti-human IgM or IgG-labeled with fluorescein-isothiocyanate (FITC) (Southern Biotechnology Associates, Inc., Birmingham, AL) was used per 2×10^5 cells.

Complement-dependent cytotoxicity (CDC): Complement-dependent cytotoxicity was assayed at a serum dilution of 1:10 with MCF-7 cells and human complement by chromium-release assay as previously described.[16] All assays were performed in triplicates. Controls included cells incubated only with culture medium, complement, antisera, or mAb MBr1. Spontaneous release was the chromium released by target cells incubated with complement alone. The maximum release was the amount of ^{51}Cr released from target cells lysed with 1% Triton-100. Percent cytolysis was calculated according to the formula: specific release (%) = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

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[1] A. Lanzavecchia, *Science* **1993**, *260*, 937–944.
[2] D. M. Pardoll, *Curr. Opin. Immunol.* **1993**, *5*, 719–725.
[3] N. R. Rabinovich, P. McInnes, D. L. Klein, B. F. Hall, *Science* **1994**, *265*, 1401–1404.
[4] P. O. Livingston, *Immunol. Rev.* **1995**, *145*, 147–166.
[5] R. R. Koganty, M. A. Reddish, B. M. Longenecker in *Glycopeptides and Related Compounds. Synthesis, Analysis and Applications* (Eds.: D. G. Large, C. D. Warren), Marcel Dekker, New York, **1997**, pp. 707–743.
[6] E. G. Bremer, S. B. Levery, S. Sonnino, R. Ghidoni, S. Canevari, R. Kannagi, S. Hakomori, *J. Biol. Chem.* **1984**, *259*, 14773–14777.
[7] S. Menard, E. Tagliabue, S. Canevari, G. Fossati, M. I. Colnaghi, *Cancer Res.* **1983**, *43*, 1295–1300.
[8] V. Kudryashov, G. Ragupathi, I. J. Kim, M. E. Breimer, S. J. Danishefsky, P. O. Livingston, K. O. Lloyd, *Glycoconjugate J.* **1998**, *15*, 243–249.
[9] S. Zhang, C. Cordon-Cardo, H. S. Zhang, V. E. Reuter, S. Adluri, W. B. Hamilton, K. O. Lloyd, P. O. Livingston, *Int. J. Cancer* **1997**, *73*, 42–49.
[10] T. K. Park, I. J. Kim, S. Hu, M. T. Bilodeau, J. T. Randolph, O. Kwon, S. J. Danishefsky, *J. Am. Chem. Soc.* **1996**, *118*, 11488–11500.
[11] S. J. Danishefsky, M. T. Bilodeau, *Angew. Chem.* **1996**, *108*, 1482–1522; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1380–1419.
[12] G. Ragupathi, T. K. Park, S. Zhang, I. J. Kim, L. Graber, S. Adluri, K. O. Lloyd, S. J. Danishefsky, P. O. Livingston *Angew. Chem.* **1997**, *109*, 166–169; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 125–128.
[13] I. J. Kim, T. K. Park, S. Hu, S. J. Danishefsky, P. O. Livingston, S. Zhang, *J. Org. Chem.* **1995**, *60*, 7716–7717.
[14] P. O. Livingston, G. Y. C. Wong, S. Adluri, Y. Tao, M. Padavan, R. Parente, C. Hanlon, F. Helling, G. Ritter, H. F. Oettgen, L. J. Old, *J. Clin. Oncol.* **1994**, *12*, 1036–1044.
[15] G. Ragupathi, R. R. Koganty, D. X. Qui, K. O. Lloyd, P. O. Livingston, *Glycoconjugate J.* **1998**, *15*, 217–221.
[16] F. Helling, A. Zhang, A. Shang, S. Adluri, M. Calves, R. R. Koganty, B. M. Longenecker, H. F. Oettgen, P. O. Livingston, *Cancer Res.* **1995**, *55*, 2783–2788.

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